INVENTION TITLE

Secreted Insecticidal Protein and Gene Compositions from Bacillus thuringiensis and Uses Therefor

DESCRIPTION

[Para 1] Background of Invention

[Para 2] The present invention relates to a new family of genes encoding lepidopteran-toxic proteins and insecticidal fragments thereof. In particular, the present invention is directed to exemplary proteins designated herein as TIC900, TIC402, TIC403, TIC404, TIC961, TIC962, TIC963, TIC965 and TIC966, and insecticidal fragments thereof, each encoded by exemplary nucleotide coding sequences designated herein respectively as *tic*900, *tic*402, *tic*403, *tic*404, *tic*434, *tic*961, *tic*962, *tic*963, *tic*965, and *tic*966, as well as to nucleotide sequence homologs that (1) encode insecticidal proteins and (2) hybridize to the *tic*900, *tic*402, *tic*403, *tic*404, *tic*434, *tic*961, *tic*962, *tic*963, *tic*965, and *tic*966 coding sequences under stringent hybridization conditions. The present invention also relates to host cells transformed with one or more nucleotide sequences of the present invention or transformed with variants of the nucleotide sequences set forth herein, genes related by identity and/or similarity to the sequences set forth herein, and/or homologs thereof, particularly those sequences that have been modified for improved expression in plants. In a preferred embodiment, the transformed host cells are plant cells.

[Para 3] Almost all field crops, plants, and commercial farming areas are susceptible to attack by one or more insect pests. Particularly problematic are Coleopteran and Lepidoptern pests. For example, vegetable and cole crops such as artichokes, kohlrabi, arugula, leeks, asparagus, lentils, beans, lettuce (e.g., head, leaf, romaine), beets, bok choy, malanga, broccoli, melons (e.g., muskmelon, watermelon, crenshaw, honeydew, cantaloupe), brussels sprouts, cabbage, cardoni, carrots, napa, cauliflower, okra, onions, celery, parsley, chick peas, parsnips, chicory, peas, chinese cabbage, peppers, collards, potatoes, cucumber, pumpkins, cucurbits, radishes, dry bulb onions, rutabaga, eggplant, salsify, escarole, shallots, endive, soybean, garlic, spinach, green onions, squash, greens, sugar beets, sweet potatoes, turnip, swiss chard, horseradish, tomatoes, kale, turnips, and a variety of spices are sensitive to infestation by one or more of the following insect pests: alfalfa looper, armyworm, beet armyworm, artichoke plume moth, cabbage budworm, cabbage looper, cabbage webworm, corn earworm, celery leafeater, cross-striped cabbageworm, european corn borer, diamondback moth, green cloverworm, imported cabbageworm, melonworm, omnivorous leafroller, pickleworm, rindworm complex, saltmarsh caterpillar, soybean looper, tobacco budworm, tomato fruitworm, tomato hornworm, tomato pinworm, velvetbean caterpillar, and yellowstriped armyworm. Likewise, pasture and hay crops such as alfalfa, pasture grasses and silage are often attacked by such pests as armyworm, beef armyworm, alfalfa caterpillar, European skipper, a variety of loopers and webworms, as well as yellowstriped armyworms.

[Para 4] Fruit and vine crops such as apples, apricots, cherries, nectarines, peaches, pears, plums, prunes, quince almonds, chestnuts, filberts, pecans, pistachios, walnuts, citrus, blackberries, blueberries, boysenberries, cranberries, currants, loganberries, raspberries, strawberries, grapes, avocados, bananas, kiwi, persimmons, pomegranate, pineapple, and tropical fruits are often susceptible to attack and defoliation by achema sphinx moth, amorbia, armyworm, citrus cutworm,

banana skipper, blackheaded fireworm, blueberry leafroller, cankerworm, cherry fruitworm, citrus cutworm, cranberry girdler, eastern tent caterpillar, fall webworm, fall webworm, filbert leafroller, filbert webworm, fruit tree leafroller, grape berry moth, grape leaffolder, grapeleaf skeletonizer, green fruitworm, gummosos-batrachedra commosae, gypsy moth, hickory shuckworm, hornworms, loopers, navel orangeworm, obliquebanded leafroller, omnivorous leafroller. omnivorous looper, orange tortrix, orangedog, oriental fruit moth, pandemis leafroller, peach twig borer, pecan nut casebearer, redbanded leafroller, redhumped caterpillar, roughskinned cutworm, saltmarsh caterpillar, spanworm, tent caterpillar, thecla-thecla basillides, tobacco budworm, tortrix moth, tufted apple budmoth, variegated leafroller, walnut caterpillar, western tent caterpillar, and yellowstriped armyworm.

[Para 5] Field crops such as canola/rape seed, evening primrose, meadow foam, corn (field, sweet, popcorn), cotton, hops, jojoba, peanuts, rice, safflower, small grains (barley, oats, rye, wheat, etc.), sorghum, soybeans, sunflowers, and tobacco are often targets for infestation by insects including armyworm, asian and other corn borers, banded sunflower moth, beet armyworm, bollworm, cabbage looper, corn rootworm (including southern and western varieties), cotton leaf perforator, diamondback moth, european corn borer, green cloverworm, headmoth, headworm, imported cabbageworm, loopers (including Anacamptodes spp.), obliquebanded leafroller, omnivorous leaftier, podworm, podworm, saltmarsh caterpillar, southwestern corn borer, soybean looper, spotted cutworm, sunflower moth, tobacco budworm, tobacco hornworm, and velvetbean caterpillar.

[Para 6] Bedding plants, flowers, ornamentals, vegetables and container stock are frequently fed upon by a host of insect pests such as armyworm, azalea moth, beet armyworm, diamondback moth, ello moth (hornworm), Florida fern caterpillar, Io moth, loopers, oleander moth, omnivorous leafroller, omnivorous looper, and tobacco budworm.

[Para 7] Forests, fruit, ornamental, and nut-bearing trees, as well as shrubs and other nursery stock are often susceptible to attack from diverse insects such as bagworm, blackheaded budworm, browntail moth, california oakworm, douglas fir tussock moth, elm spanworm, fall webworm, fruittree leafroller, greenstriped mapleworm, gypsy moth, jack pine budworm, mimosa webworm, pine butterfly, redhumped caterpillar, saddleback caterpillar, saddle prominent caterpillar, spring and fall cankerworm, spruce budworm, tent caterpillar, tortrix, and western tussock moth. Likewise, pests such as armyworm, sod webworm, and tropical sod webworm often attack turf grasses.

[Para 8] Because crops of commercial interest are often the target of insect attack, environmentally-sensitive methods for controlling or eradicating insect infestation are desirable in many instances. This is particularly true for farmers, nurserymen, growers, and commercial and residential areas which seek to control insect populations using eco-friendly compositions.

[Para 9] *Bacillus thuringiensis* is a gram-positive bacterium that produces proteinaceous crystalline inclusions during sporulation. These *B. thuringiensis* crystal proteins are often highly toxic to specific insects. Insecticidal activities have been identified for crystal proteins from various *B. thuringiensis* strains against insect larvae from the insect orders Lepidoptera (caterpillars), Coleoptera (beetles) and Diptera (mosquitoes, flies).

[Para 10] Individual *B. thuringiensis* crystal proteins, also called delta-endotoxins or parasporal crystals or toxin proteins, can differ extensively in their structures and insecticidal activities. These insecticidal proteins are encoded by genes typically located on large plasmids, greater than 30 mega Daltons (mDa) in size, that are found in *B. thuringiensis* strains. A number of these *B. thuringiensis* toxin genes have been cloned and the insecticidal crystal protein products characterized for their

specific insecticidal properties. Hofte et al. (1989) and Schnepf et al. (1998) provide reviews of *B. thuringiensis* toxin genes and crystal proteins.

[Para 11] The insecticidal properties of *B. thuringiensis* have been long recognized, and *B. thuringiensis* strains have been incorporated in commercial biological insecticide products for over forty years. Commercial *B. thuringiensis* insecticide formulations typically contain dried sporulated *B. thuringiensis* fermentation cultures whose crystal proteins are toxic to various insect species.

[Para 12] Traditional commercial *B. thuringiensis* bio-insecticide products are derived from "wild-type" *B. thuringiensis* strains, i.e., purified cultures of *B. thuringiensis* strains isolated from natural sources. Newer commercial *B. thuringiensis* bio-insecticide products are based on genetically altered *B. thuringiensis* strains, such as the transconjugant *B. thuringiensis* strains described in U.S. Patent Nos. 5,080,897 and 4,935,353.

[Para 13] A characteristic of crystal proteins is their ability to coalesce to form crystals inside the *B. thuringiensis* mother cell. Upon lysis of the mother cell the proteins are released as crystals into the external environment. In addition, *B. thuringiensis* also produces non-crystal proteins that, in contrast to crystal proteins, are secreted by *B. thuringiensis* cells as soluble proteins into the culture medium. Secreted non-crystal proteins of *B. thuringiensis* include phospholipases, proteases, and β-lactamase that have little, if any, insecticidal activity. However, three secreted non-crystal proteins of *B. thuringiensis* designated Vip1, Vip2 and Vip3 have been reported to be toxic to coleopteran or lepidopteran insects (Estruch et al., 1996; U. S. Patent No. 5,866,326; WO94/21795; WO96/10083). A non-crystal protein of *B. thuringiensis* designated CryV is reported to be toxic to lepidopteran insects (Kostichka et al., 1996). A large number of *Bacillus thuringiensis* isolates producing extracellular secreted insecticidal toxin proteins have been identified by a number of different investigators. Such isolates have all been shown to produce one or more of these VIP or CryV toxin proteins or closely related homologs. Coleopteran inhibitory secreted BT proteins such as TIC901, TIC1201, TIC407, and TIC417 have been previously disclosed but appear to be unrelated to the proteins of the present invention (US Provisional Patent Application No. 60/485,483 filed July 7, 2003; PCT/US04/21692 filed July 6, 2004).

[Para 14] The inventors herein disclose a new class of extracellular secreted insecticidal protein toxins that do not exhibit homology to the known VIP or CryV classes of proteins. None of the one hundred thirty-seven known insect-toxic proteins of *B. thuringiensis* (Crickmore et al., 1998), more or less, are substantially related to the proteins of the present invention. In fact, no significant homology was found between the sequences of the proteins of the present invention and any of the thousands of protein sequences contained in the National Center for Genome Resources (GenBank), Santa Fe, NM.

[Para 15] Summary of Invention

[Para 16] In one embodiment, the present invention relates to an isolated and purified insecticidal protein, exhibiting an amino acid sequence substantially as set forth in SEQ ID NO:4, (TIC900), SEQ ID NO:6 (TIC402), SEQ ID NO:8 (TIC403), SEQ ID NO:10 (TIC404), SEQ ID NO:30 (TIC434), SEQ ID NO:12 (TIC961), SEQ ID NO:14 (TIC962), SEQ ID NO:16 (TIC963), SEQ ID NO:18 (TIC965), and SEQ ID NO:20 (TIC966), or related amino acid sequences and homologs thereof. Insecticidal activity of TIC900 and related proteins have been demonstrated in

bioassays with lepidopteran insects including European corn borer (ECB), tobacco budworm (TBW) and Diamondback Moth (DBM), as shown herein.

[Para 17] In another embodiment, the present invention relates to an isolated and purified nucleotide sequence, i.e. a coding sequence, comprising a nucleotide sequence as set forth in SEQ ID NO:3 (*tic*900), SEQ ID NO:5 (*tic*402), SEQ ID NO:7 (*tic*403), SEQ ID NO:9 (*tic*404), SEQ ID NO:29 (*tic*434), SEQ ID NO:11 (*tic*961), SEQ ID NO:13 (*tic*962), SEQ ID NO:15 (*tic*963), SEQ ID NO:17 (*tic*965), or SEQ ID NO: 19 (*tic*966), or related sequences or homologs thereof. The native *tic*900 coding sequence as set forth in SEQ ID NO:3 encodes the TIC900 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:4. Organisms producing TIC900 or related proteins exhibit insecticidal activity and/or insect-resistance properties. The native *tic*402 coding sequence as set forth in SEQ ID NO:5 encodes the TIC402 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:6. The native *tic*403 coding sequence as set forth in SEQ ID NO:7 encodes the TIC403 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:8. The native *tic*404 coding sequence as set forth in SEQ ID NO:9 encodes the TIC404 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:10. The native *tic*434 coding sequence as set forth in SEQ ID NO:29 encodes the TIC434 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:30. The native *tic*961 coding sequence as set forth in SEQ ID NO:11 encodes the TIC961 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:12. The native *tic*962 coding sequence as set forth in SEQ ID NO:13 encodes the TIC962 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:14. The native *tic*963 coding sequence as set forth in SEQ ID NO:15 encodes the TIC963 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:16. The native *tic*965 coding sequence as set forth in SEQ ID NO:17 encodes the TIC965 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:18. The native *tic*966 coding sequence as set forth in SEQ ID NO:19 encodes the TIC966 protein exhibiting the amino acid sequence as set forth in SEQ ID NO:20. TIC900 or related proteins and nucleotide sequences derived from Bt strains that encode these proteins are described herein as homologs of each other, i.e., insecticidal proteins or insecticidal fragments thereof encoded by nucleotide sequences that hybridize to each or any of the sequences disclosed herein either under specific hybridization conditions or under stringent hybridization conditions, and are specifically intended to be included within the scope of the present invention.

[Para 18] In a further embodiment, the present invention relates to a biologically pure culture of a *Bacillus thuringiensis* bacterium transformed with a plasmid vector containing a nucleotide sequence as set forth in SEQ ID NO:3 (*tic*900), SEQ ID NO:5 (*tic*402), SEQ ID NO:7 (*tic*403), SEQ ID NO:9 (*tic*404), SEQ ID NO:29 (*tic*434), SEQ ID NO:11 (*tic*961), SEQ ID NO:13 (*tic*962), SEQ ID NO:15 (*tic*963), SEQ ID NO:17 (*tic*965), or SEQ ID NO: 19 (*tic*966), or a related sequence or homolog that produces an insecticidal protein and secretes the protein into the extracellular space surrounding the bacterial strain during fermentation. An exemplary strain SIC9002 has been deposited in the Northern Regional Research Laboratory of Agricultural Research Service Center Collection (NRRL), USDA, 1815 North University Street, Peoria, IL 61604, pursuant to the Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure on April 25, 2000 and has been assigned the accession No. NRRL B-30582. One plasmid containing the *tic*900 nucleotide sequence is set forth herein as pBD1.

[Para 19] In a further embodiment, the invention also relates to a biologically pure culture of a *B. thuringiensis* bacterium designated as strain EG5438 exhibiting insecticidal activity against lepidopteran insects. *B. thuringiensis* strain EG5438 represents a wild type *B. thuringiensis* strain from which a *tic*900 coding sequence was isolated. The strain has been deposited in the NRRL, USDA, pursuant to the Budapest Treaty on May 3, 2000 and has been assigned the accession No. NRRL B-30584.

[Para 20] In a further embodiment, the present invention provides a nucleotide sequence as set forth in SEQ ID NO:3 encoding a TIC900 amino acid sequence (SEQ ID NO:4), and an oligonucleotide portion that can be labeled and used as a hybridization probe for identifying additional related genes encoding related insecticidal proteins or homologues thereof. Other related nucleotide sequences specifically exemplified herein comprise sequences as set forth in SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:29, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19, each of which encode insecticidal protein toxins as set forth in SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:30, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20, respectively.

[Para 21] In yet a further embodiment, the invention provides plant cells and plants that have been transformed with a nucleotide sequence encoding a TIC900 or related protein as set forth in SEQ ID NO:4 or insecticidal fragment thereof, or a TIC900 protein homolog thereof, selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:30, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20. The nucleotide sequence can be translated and expressed by plant cells and in plant tissues at levels sufficient to inhibit or kill lepidopteran insect pests that come into contact with the transgenic plant expressing said protein, particularly when said pests ingest parts of said transgenic plant. Both monocot and dicot plants are within the scope of the invention. Modification of the sequence may be required in order to affect the maximum level of expression and to enhance the ability of the plant containing the sequence to produce insecticidal levels of the TIC900 or related protein. Transformation of plants with the nucleotide sequences disclosed herein may result in increased frequency of transformants that express the transgene, i.e., *tic*900 or its homolog, as well as the generation of a greater percentage of transformation events exhibiting morphologically normal physiology.

[Para 22] In yet a further embodiment, the present invention also provides a method for producing a transgenic plant that exhibits increased expression levels of a nucleotide sequence encoding a TIC900 protein or insecticidal fragment thereof or its homolog and thereafter increased levels of the insecticidal TIC900 protein or its homolog. Thus the plants transformed with the nucleotide sequences disclosed herein exhibit improved and increased levels of lepidopteran pest resistance abilities in comparison to a plant lacking a nucleotide sequence encoding a TIC900, an insecticidal fragment of a TIC900, or one of its homologs.

[Para 23] In accomplishing the foregoing, a method for expressing a nucleotide sequence encoding a TIC900 protein or its homolog in a plant is provided comprising the steps of a) inserting into the genome of a plant cell a nucleic acid sequence comprising in the 5' to 3' direction, a plant functional promoter operably linked to a structural DNA sequence optimized for plant expression that causes production of an RNA sequence encoding all of or an insecticidal fragment of a TIC900 polypeptide sequence as set forth in SEQ ID NO:4, or its homolog selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:30, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20, or a sequence having at least from about 80%, or from at least about 85%, or from at least about 90%, or from at least about 95%, or from at least about 99% sequence identity to the amino acid sequence as set forth in SEQ ID NO:4, or a sequence encoding an insecticidal protein that hybridizes to any of these sequences under either specific or stringent hybridization conditions, and a 3' non-translated DNA sequence that functions in the cells of the plant to cause transcription termination and polyadenylation; b) obtaining transformed plant cells containing the nucleic acid sequence; and c) generating from the transformed plant cells genetically transformed plants that express the nucleotide sequence encoding the TIC900 or a related protein, wherein the transformed plants are morphologically normal and exhibit elevated or improved levels of lepidopteran pest resistance compared to a plant not transformed to express said protein.

[Para 24] Another embodiment of the present invention is the provision for antibodies that bind specifically to epitopes presented only by the TIC900 protein or its homologs. Antibodies can be used for identifying the presence of a TIC900 protein or a homolog, for purifying the protein or homolog, for identifying a nucleotide sequence from which a TIC900 protein or a homolog is being expressed, and for use in kits designed to allow the detection of a TIC900 protein or a homolog or the detection of a nucleotide sequence expressing the protein or homolog.

[Para 25] The inventors contemplate that the protein compositions disclosed herein will find particular utility as insecticides for topical and/or systemic application to field crops, grasses, fruits and vegetables, and ornamental plants. In a preferred embodiment, the bioinsecticide composition comprises an oil flowable suspension of bacterial cells which expresses a novel insecticidal protein disclosed herein. Preferably the cells are *B. thuringiensis* EG5438 or SIC9002 cells, however, any such bacterial host cell expressing the novel nucleic acid segments disclosed herein and producing a crystal protein is contemplated to be useful, such as *B. megaterium*, *B. subtilis*, *E. coli*, or *Pseudomonas* spp.

[Para 26] A particular advantage of the present invention comprises an improvement in insect resistance management (IRM). The ability to combine two or more insecticidal agents, each toxic to the same insect pest species, into a single composition, and each agent exhibiting a mode of action different from the other insecticidal agents with which it is combined, present a means for more effectively controlling a particular insect pest species by substantially reducing the likelihood that resistance to the insecticidal composition will develop in a population. The TIC900 protein an insecticidal fragment thereof, or any homolog thereof, of the present invention can be combined with any number of known insecticidal agents to achieve the level of resistance management in a particular composition, preferably by expression of the combination of insecticidal agents in plants. In particular TIC900 or related insecticidal protein compositions can be combined with a Cry1 or Cry2 amino acid sequence or a variant thereof to achieve control of various lepidopteran plant pest species, or with other appropriate Cry proteins, and with various insecticidal compositions derived from *Xenorhabdus* and *Photorhabdus* bacterium species that have been shown to exhibit insecticidal bioactivity directed to lepidopteran plant pest species. Preferably the *in planta* use of these compositions would be directed to enhanced expression of the proteins in the parts of the plant that exhibit the greatest vulnerability to lepidopteran insect predation. For protection of maize species against European corn borer (ECB), it would be preferable to achieve the highest levels of expression in the leaves and stems of the plant. For tobacco species susceptible to budworm, it would be preferable to achieve the highest levels of expression in the sprouting parts of the plant, i.e., within the bud systems of the plant. For protection of a cruciferous vegetable species against diamondback moth (DBM), it would be preferable to achieve the highest levels of expression in the leaves and stems of the plant.

[Para 27] The insecticidal proteins of the present invention can also be combined with insecticidal and/or fungicidal toxins expressed *in planta* to achieve a recombinant plant that exhibits multiple levels of resistance to infestation by pests that are not beneficial to plants. For example, a protein of the present invention can be expressed along with a protein that exhibits coleopteran insect control, and/or along with a protein or other agent that exhibits antifungal activity, to achieve a recombinant transgenic plant that exhibits improved resistance to lepidopteran insect pests, coleopteran insect pests, and fungal pests. Other permutations of levels of resistance are known to those of skill in the art, such as means for resistance to piercing and sucking insect infestation, and nematode infestation, etc. The insecticidal proteins of the present invention can also be combined with one or more nucleotide sequences expressed as one or more dsRNA's for use in suppression of one or more genes (1) in the target pest as a means for achieving a plant that exhibits multiple layers of resistance to infestation by a particular pest, (2) in the plant as a means for achieving desired plant traits, or (3) in various combinations to achieve the desired properties of (1) or (2) collectively.

[Para 28] Chimeric proteins consisting of all or a part of one or more proteins of the present invention fused to other proteins that are useful in plant protection from infestation or otherwise are contemplated herein. For example, domains of the proteins of the present invention have been found to exhibit a low level of similarity to other Bt toxins, such as Cry3Aa toxin domain I, Cry1Ca toxin domain II, and Cry1Ja toxin domain III (in particular, Domains I, II, and III of the toxin portion of the TIC900 protein, respectively). The proteins of the present invention can be fused to the protoxin domains of any of the Cry1 proteins known in the art, resulting in crystal toxin protein formation when expressed in Bt or other Bacillus strains of bacteria. Furthermore, the domains identified herein within the amino acid sequence of the proteins of the present invention can be exchanged with other similar domains from insecticidal Bt toxin proteins to achieve improved insecticidal activity and/or host ranges that have not previously been observed with Cry1 toxin domain exchanges (Malvar et al. US Patent No. 6,017,534; Galizzi et al, PCT/EP90/0114, WO 91/01087).

[Para 29] Another embodiment comprises an isolated polynucleotide that encodes a *Bacillus thuringiensis* insecticidal toxin or insecticidal fragment thereof, active against an insect pest, wherein the toxin or insecticidal fragment has a molecular weight between approximately 65,000 Daltons and approximately 70,000 Daltons. In addition, the nucleotide sequence encoding the toxin, or the complement thereof, hybridizes under specific or stringent hybridization conditions to SEQ ID NO:3. The toxin preferably exhibits biological activity in controlling or killing a lepidopteran insect pest, preferably European corn borer (ECB), tobacco budworm (TBW) and/or diamondback moth (DBM). In one embodiment the nucleotide sequence encoding the toxin is optimized for expression in plants, yet encodes substantially the toxin or an insecticidal fragment thereof, i.e., encodes the same or substantially the same amino acid sequence as present in the native amino acid sequence.

[Para 30] Another embodiment of the present invention provides for host cells transformed to contain a polynucleotide encoding an insecticidal protein of the present invention or an insecticidal fragment thereof. Preferably the nucleotide sequences of the present invention are modified to improve expression of the proteins of the present invention in a preferred host cell. The host cell of the present invention is selected from the group consisting of a bacterial cell, a fungal cell, and a plant cell. Expression in a plant cell can comprise expression to achieve accumulation of the insecticidal protein in the cytoplasm, or can result in the insecticidal protein being accumulated into a subcellular organelle such as a plastid, chloroplast, or mitochondria. Alternatively the insecticidal protein of the present invention or insecticidal fragments thereof could be localized to the protein secretion machinery of the particular host cell and result in an accumulation of the protein product outside of the cell and into the extracellular spaces surrounding the cell.

[Para 31] An additional embodiment of the present invention provides a method for controlling infestation of a plant by a lepidopteran insect species. Preferably a pesticidal amount of an insecticidal protein of the present invention or insecticidal fragment thereof is provided for consumption by the insect pest in the diet of the insect. The diet can consist of a plant part that the insect normally feeds upon, such as a plant tissue or plant cell. The insecticidal protein or insecticidal fragment thereof can be provided in a composition that is applied to the surface of the plant tissue, plant part, or plant cell or more preferably can be produced by the protein synthesis machinery of the cell and, as described above, accumulated within the plant cell or secreted outside of the plant cell, so long as the amount of the protein toxin provided is an insecticidal amount sufficient to inhibit the insect pest from further feeding, or to inhibit the further growth and development of the insect pest, or to cause mortality to the insect pest. The insecticidal toxin or fragment thereof is derived from a nucleotide sequence that is encoded in *Bacillus thuringiensis* by a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence substantially complementary to SEQ ID NO:3.

[Para 32] The present invention also provides a method for detecting a first nucleotide sequence that hybridizes to a second nucleotide sequence as set forth in SEQ ID NO:3, wherein the first nucleotide sequence encodes an insecticidal protein or insecticidal fragment thereof and hybridizes under specific or stringent hybridization conditions to the second nucleotide sequence. Other exemplary second nucleotide sequences are SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:29, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19.

[Para 33] It is also contemplated that the proteins of the present invention would be useful when expressed in planta to provide an improved level of protection from insect infestation to plants expressing the proteins or insecticidal fragments thereof. Therefore it is envisioned that one or more nucleotide sequences encoding a TIC900 insecticidal protein or insecticidal fragment thereof or homolog thereof, or combinations thereof, whether expressed individually or as chimeras or as fusions, would be introduced into the plant cell, either into the genome, into the chloroplast or mitochondrial DNA, or into an organelle as a stable and autonomously replicating extra-chromosomal element, for expression of the said TIC900 protein or insecticidal fragment thereof or homolog thereof. Preferably the sequence is a non-naturally occurring nucleotide sequence that encodes the insecticidal protein or insecticidal fragment thereof. Plant cells transformed with such sequences are provided for herein. Plants grown from the transformed plant cells are provided by the instant inventions. Seeds and progeny of the seeds from the transformed plants of the present invention are also provided so long as the seeds contain at least the sequences encoding the insecticidal proteins or insecticidal protein fragments thereof. The nucleotide sequences envisioned are at least from about 60 to about 85% identical to the nucleotide sequences of the present invention as isolated from *B. thuringiensis*.

[Para 34] Exemplary sequences of the present invention include at least, in addition to those related to SEQ ID NO:5 and SEQ ID NO:4: (1) the nucleotide sequence as set forth in SEQ ID NO:5, and the amino acid sequence encoded by SEQ ID NO:5 as set forth in SEQ ID NO:6, also referred to herein as insecticidal protein TIC402; (2) the nucleotide sequence as set forth in SEQ ID NO:7, and the amino acid sequence encoded by SEQ ID NO:7 as set forth in SEQ ID NO:8, also referred to herein as insecticidal protein TIC403; (3) the nucleotide sequence as set forth in SEQ ID NO:9, and the amino acid sequence encoded by SEQ ID NO:9 as set forth in SEQ ID NO:10, also referred to herein as insecticidal protein TIC404; (4) the nucleotide sequence as set forth in SEQ ID NO:29, and the amino acid sequence encoded by SEQ ID NO:29 as set forth in SEQ ID NO:30, also referred to herein as insecticidal protein TIC434; (5) the nucleotide sequence as set forth in SEQ ID NO:11, and the amino acid sequence encoded by SEQ ID NO:11 as set forth in SEQ ID NO:12, also referred to herein as insecticidal protein TIC961; (6) the nucleotide sequence as set forth in SEQ ID NO:13, and the amino acid sequence encoded by SEQ ID NO:13 as set forth in SEQ ID NO:14, also referred to herein as insecticidal protein TIC962; (7) the nucleotide sequence as set forth in SEQ ID NO:15, and the amino acid sequence encoded by SEQ ID NO:15 as set forth in SEQ ID NO:16, also referred to herein as insecticidal protein TIC963; (8) the nucleotide sequence as set forth in SEQ ID NO:17, and the amino acid sequence encoded by SEQ ID NO:17 as set forth in SEQ ID NO:18, also referred to herein as insecticidal protein TIC965; and (9) the nucleotide sequence as set forth in SEQ ID NO:19, and the amino acid sequence encoded by SEQ ID NO:19 as set forth in SEQ ID NO:20, also referred to herein as insecticidal protein TIC966. Each of these proteins and the native *B.t.* nucleotide sequences encoding these proteins are related to TIC900 as defined herein. For example, and respectively, SEQ ID NO:5 is a nucleotide sequence encoding a TIC402 insecticidal protein as set forth in SEQ ID NO:6. SEQ ID NO:5 as shown herein is identifiable by hybridization to SEQ ID NO:3 under stringent conditions. SEQ ID NO:5 encodes a protein that exhibits lepidopteran toxic biological activity, exhibiting toxicity to European corn borer (ECB), tobacco budworm (TBW) and/or diamondback moth (DBM). SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:29, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19 are each capable of hybridizing to each other under stringent conditions, and each sequence can be identified by hybridization to SEQ ID NO:3 under stringent conditions, and each sequence can be identified by amplification using the

oligonucleotide primers as set forth in SEQ ID NO:21 and SEQ ID NO:22. The primers as set forth in SEQ ID NO:21 and SEQ ID NO:22 are diagnostic for identifying the presence of a nucleotide sequence encoding a TIC900 or related insecticidal protein in a sample. These oligonucleotides, when used together under defined amplification conditions and in the presence of a suitable nucleotide sequence substrate, produce an amplicon that is diagnostic for the presence of a TIC900 coding sequence or a homolog thereof. This particular reaction is useful for detecting the presence of a *B.t.* gene encoding an insecticidal protein corresponding to a TIC900 or related protein in a sample, and greatly simplifies the search for and identification of such related sequences.

[Para 35] Kits for detecting the presence of the nucleotide sequences of the present invention are also contemplated. Such kits contain one or more nucleotide sequences each for use as a probe for detecting the presence of a nucleotide sequence encoding an insecticidal protein of the present invention or fragment thereof. Such kits could also or alternatively contain antibody specific for binding to one or more peptides of the proteins of the present invention, as well as reagents for use with the probe or antibody, and the kits would also contain control samples for use in ensuring that the nucleotides or peptides identified with the probe and or antibody and reagents were functioning according to the manufacturers" instructions. All of the reagents necessary for carrying out the methods of identification of either nucleotide sequences or peptides would be packaged together in a kit along with instructions for use. An exemplary kit could contain a TIC900 or related nucleotide sequence encoding an insecticidal protein along with a sample of the exemplary nucleotide sequence amplification primers as set forth in SEQ ID NO:21 and SEQ ID NO:22, together with the necessary reagents necessary for carrying out an amplification reaction, all packaged together in the kit.

[Para 36] A plant or plant tissue transformed to contain (a) a nucleotide sequence encoding one or more of the proteins of the present invention, (2) all or an insecticidally active portion of one or more of the proteins of the present invention, or (3) a chimera containing all or any portion of one or more proteins of the present invention can be detected using any number of means well known in the art including but not limited to nucleotide sequence based detection methods and/or protein based detection methods. Agronomically and commercially important products and/or compositions of matter derived from such transformed plants or plant tissues include but are not limited to animal feed, commodities, and corn, soy, cotton, canola, wheat, oat, rice, sugar-cane, chick-pea, and cow-pea products and by-products that are intended for use as food for human consumption or for use in compositions that are intended for human consumption including but not limited to flours, meals, syrups, oil, starch, popcorn, cakes, cereals containing the fruits and seeds of these crops and by-products, and the like are intended to be within the scope of the present invention if these products and compositions of matter contain detectable amounts of the nucleotide sequences encoding the proteins or derivatives of the proteins as set forth herein.

[Para 37] Plants or plant parts suspected of containing a protein or nucleotide encoding a protein of the present invention in a biological sample can be detected using the method comprising the steps of contacting a sample suspected of containing said nucleotide with a polynucleotide probe that hybridizes under stringent hybridization conditions with said nucleotide and that does not hybridize under stringent hybridization conditions with a nucleotide from a control plant, subjecting said sample and said probe to said stringent hybridization conditions, and detecting the hybridization of said probe to the nucleotide.

[Para 38] One embodiment of the present invention comprises a biological sample derived from a transgenic plant, tissue, or seed, wherein the sample comprises a nucleotide sequence which is or is complementary to a sequence encoding a protein of the present invention, and wherein said sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method.

The sample can consist of a sample that is selected from the group consisting of an extract obtainable from the transgenic plant containing the nucleotide sequence, and the extract can contain any nucleotide sequence encoding one or more of the proteins of the present invention, or the complement thereof. The biological sample is preferably selected from the group consisting of a flour such as corn flour, a meal such as corn meal, a syrup such as corn syrup, an oil such as corn oil, cotton oil, linseed oil, soybean or canola oil, safflower oil, sunflower oil, peanut oil, and the like, a starch such as corn starch, and any cereal that can be manufactured in whole or in part to contain grain or grain by-products. The nucleotide sequence is detectable in the extract using a nucleic acid amplification or nucleic acid hybridization method.

[Para 39] Brief Description Of The Sequences

[Para 40] SEQ ID NO:1 represents an amino acid sequence deduced by Edmund degradation of a 14 kDa cyanogen bromide fragment of a TIC900 protein and corresponds to amino acid positions 397-414 as set forth in SEQ ID NO:4.

[Para 41] SEQ ID NO:2 represents the nucleotide sequence of a hybridization probe designated as WD470 designed based upon the amino acid sequence as set forth in SEQ ID NO:1, for use in detecting nucleotide sequences encoding TIC900 and related proteins.

[Para 42] SEQ ID NO:3 represents a native *Bacillus thuringiensis* nucleotide sequence consisting of 1803 consecutive nucleotides encoding a TIC900 insecticidal protein consisting of 601 amino acid as set forth in SEQ ID NO:4.

[Para 43] SEQ ID NO:4 represents the TIC900 amino acid sequence deduced from the nucleotide sequence as set forth in SEQ ID NO:3.

[Para 44] SEQ ID NO:5 represents a *tic*900 homologous nucleotide sequence encoding a native *Bacillus thuringiensis* TIC900 related protein, designated herein as TIC402.

[Para 45] SEQ ID NO:6 represents the TIC402 amino acid sequence deduced from the nucleotide sequence as set forth in SEQ ID NO:5.

[Para 46] SEQ ID NO:7 represents a *tic*900 homologous nucleotide sequence encoding a native *Bacillus thuringiensis* TIC900 related protein, designated herein as TIC403.

[Para 47] SEQ ID NO:8 represents the TIC403 amino acid sequence deduced from the nucleotide sequence as set forth in SEQ ID NO:7.

[Para 48] SEQ ID NO:9 represents a *tic*900 homologous nucleotide sequence encoding a native *Bacillus thuringiensis* TIC900 related protein, designated herein as TIC404.

[Para 49] SEQ ID NO:10 represents the TIC404 amino acid sequence deduced from the nucleotide sequence as set forth in SEQ ID NO:9.

[Para 50] SEQ ID NO:11 represents a *tic*900 homologous nucleotide sequence encoding a native *Bacillus thuringiensis* TIC900 related protein, designated herein as TIC961.

[Para 51] SEQ ID NO:12 represents the TIC961 amino acid sequence deduced from the nucleotide sequence as set forth in SEQ ID NO:11.

[Para 52] SEQ ID NO:13 represents a *tic*900 homologous nucleotide sequence encoding a native *Bacillus thuringiensis* TIC900 related protein, designated herein as TIC962.

[Para 53] SEQ ID NO:14 represents the TIC962 amino acid sequence deduced from the nucleotide sequence as set forth in SEQ ID NO:13.

[Para 54] SEQ ID NO:15 represents a *tic*900 homologous nucleotide sequence encoding a native *Bacillus thuringiensis* TIC900 related protein, designated herein as TIC963.

[Para 55] SEQ ID NO:16 represents the TIC963 amino acid sequence deduced from the nucleotide sequence as set forth in SEQ ID NO:15.

[Para 56] SEQ ID NO:17 represents a *tic*900 homologous nucleotide sequence encoding a native *Bacillus thuringiensis* TIC900 related protein, designated herein as TIC965.

[Para 57] SEQ ID NO:18 represents the TIC965 amino acid sequence deduced from the nucleotide sequence as set forth in SEQ ID NO:17.

[Para 58] SEQ ID NO:19 represents a *tic*900 homologous nucleotide sequence encoding a native *Bacillus thuringiensis* TIC900 related protein, designated herein as TIC966.

[Para 59] SEQ ID NO:20 represents the TIC966 amino acid sequence deduced from the nucleotide sequence as set forth in SEQ ID NO:19.

[Para 60] SEQ ID NO:21 represents a 5' end sequence primer used as a probe that binds specifically to TIC900 homologous sequences.

[Para 61] SEQ ID NO:22 represents a 3' end sequence primer used as a probe that binds specifically to TIC900 homologous sequences.

[Para 62] SEQ ID NO:23 represents a *tic*109 nucleotide sequence encoding a TIC109 chimeric protein consisting of a nucleotide sequence encoding a TIC900 insecticidal protein domain linked in frame to a nucleotide sequence encoding a Cry1Ac protoxin domain fragment.

[Para 63] SEQ ID NO:24 represents a TIC109 chimeric protein amino acid sequence consisting of a TIC900 insecticidal amino acid sequence (1-603) linked to a Cry1Ac protoxin domain fragment amino acid sequence (606-1168).

[Para 64] SEQ ID NO:25 represents a *tic*110 nucleotide sequence encoding a TIC110 chimeric protein consisting of a nucleotide sequence encoding a Cry1F toxin domain I fragment (nucleotides 1-723) linked in frame to a nucleotide sequence encoding a TIC900 toxin fragment domain II-III (nucleotides 724-1809) linked in frame to a nucleotide sequence encoding a Cry1Ac protoxin domain fragment (nucleotides 1810-3510).

[Para 65] SEQ ID NO:26 represents a TIC110 chimeric protein amino acid sequence consisting of a Cry1F toxin domain I fragment (amino acids 1-233) linked to a TIC900 toxin domain II-III fragment (amino acids 234-603) linked to a Cry1Ac protoxin domain fragment (amino acids 604-1170).

[Para 66] SEQ ID NO:27 represents a *tic*111 nucleotide sequence encoding a TIC111 chimeric protein consisting of a nucleotide sequence encoding a Cry1Ac toxin domain I fragment (nucleotides 1-705) linked in frame to a nucleotide sequence encoding a TIC900 toxin domain II-III fragment (nucleotides 706-1815) linked in frame to a nucleotide sequence encoding a Cry1Ac protoxin domain fragment (nucleotides 1822-3516).

[Para 67] SEQ ID NO:28 represents a TIC111 chimeric protein amino acid sequence consisting of a Cry1Ac toxin domain I fragment (amino acids 1-235) linked to a TIC900 toxin domain II-III fragment (amino acids 236-605) linked to a Cry1Ac protoxin domain fragment (amino acids 608-1172).

[Para 68] SEQ ID NO:29 represents a *B. thuringiensis* strain EG4611 about 7.5 kb nucleotide sequence containing a TIC434 coding sequence, said coding sequence being from about nucleotide position 425 through about nucleotide position 2238.

[Para 69] SEQ ID NO:30 represents a TIC434 amino acid sequence.

[Para 70] SEQ ID NO:31 represents a chimeric sequence encoding a TIC435 amino acid sequence corresponding to a TIC434 amino acid sequence fused in frame to a sequence encoding a Cry1 protoxin amino acid sequence; said TIC434 amino acid sequence coding region corresponding to about nucleotide position 1 through about nucleotide position 1825, and said Cry1 protoxin amino acid sequence coding region corresponding to about nucleotide position 1826 through about nucleotide position 3525.

[Para 71] SEQ ID NO:32 represents a chimeric TIC435 amino acid sequence.

[Para 72] Detailed Description

[Para 73] The following detailed description of the invention is provided to aid those skilled in the art in practicing the present invention. Even so, the detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed herein may be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

[Para 74] In accordance with the present invention, a new genus of nucleotide sequences encoding insecticidal proteins derived from *Bacillus thuringiensis* and related *Bacillus* strains has been discovered. As defined elsewhere herein, these nucleotide sequences all hybridize to each other under stringent conditions. The proteins encoded by these nucleotide sequences each exhibit lepidopteran species inhibitory biological activity, and so are considered to be insecticidal proteins. Each of the proteins encoded by these nucleotide sequences can be expressed in plants alone or in combinations with each other or with other lepidopteran inhibitory insecticidal agents such as proteins, crystal proteins, toxins, and/or pest specific double stranded RNA's designed to suppress genes within one or more target pests, and the like to achieve a means of insect resistance management in the field that has not feasible before by merely using the known lepidopteran insecticidal proteins derived from *Bacillus thuringiensis* strains, such as Cryl proteins and various lepidopteran inhibitory insecticidal proteins derived from *Bacillus laterosporous* species and *Bacillus sphaericus* species. The proteins of the present invention can also be used in plants in combination with other types of insecticidal toxins for achieving plants transformed to contain at least one means for controlling one or more of each of the common plant pests selected from the groups consisting of lepidopteran insect pests, coleopteran insect pests, piercing and sucking insect pests, and the like. The proteins of the present invention are also contemplated for use in formulations, either alone or in combinations with other insecticidal agents, as insecticides for topical and/or systemic application to field crops, grasses, fruits and vegetables, and ornamental plants. In a preferred embodiment, the bio-insecticide composition comprises an oil flowable suspension of bacterial cells that expresses one or more of a novel insecticidal protein disclosed herein. Preferably the cells are *B. thuringiensis* EG5438 or SIC9002 cells, however, any such bacterial host cell expressing the novel nucleic acid segments disclosed herein and producing a crystal protein

[Para 75] The insecticidal proteins of the present invention may also be used in compositions for controlling insect infestation of plants either alone or in combination with other insecticidal proteins or agents, and may also be used alone or in combination with gene suppression methodologies. As used herein "gene suppression" means any of the well-known methods for suppressing expression of protein from a gene including post transcriptional gene suppression and transcriptional suppression.

[Para 76] As used herein an "pest resistance" trait is a characteristic of a transgenic plant is resistant to attack from a plant pest such as a virus, a nematode, a larval insect or an adult insect that typically is capable of inflicting crop yield loss in a progenitor plant. Such pest resistance can arise from a natural mutation or more typically from incorporation of recombinant DNA that confers pest resistance. To impart insect resistance to a transgenic plant such recombinant DNA can, for example, encode an insect lethal protein such as a delta endotoxin of *Bacillus thuringiensis* bacteria, e.g. as is used in commercially available varieties of cotton and corn, encode an insecticidal toxin protein disclosed herein such as a TIC900 or related protein or insecticidal fragment thereof, or be transcribed to a double-stranded RNA targeted for suppression of an essential gene in the insect, or any combination of these insecticidal agents. To illustrate that the production of transgenic plants with pest resistance is a capability of those of ordinary skill in the art reference is made to U.S. Patents

5,250,515; 5,880,275 and 6,555,655 which disclose plants expressing an endotoxin of *Bacillus thuringiensis* bacteria. See also U.S. Patent 6,506,599 (Fire *et al.*) and U.S. Patent Application Publication 2003/0061626 A1 (Plaetinck *et al.*) and U.S. Patent Application Publication 2003/0150017 A1 (Mesa *et al.*) which disclose control of invertebrates by permitting the pest to feed on transgenic plants which produce double-stranded RNA for suppressing a target gene in the pest.. See also U.S. Patent 5,986,175 (Jilka *et al.*) that discloses the control of viral pests by transgenic plants which express viral replicase. All of the above-described patents and applications disclosing materials and methods for pest control in plants are incorporated herein by reference.

[Para 77] Surprisingly, the proteins of the present invention appear to be unrelated to any of the *Bacillus thuringiensis* insecticidal proteins heretofore discovered in the art. The proteins of the present invention are shown herein to be excreted into the extracellular space surrounding the *Bacillus* species from which they are derived. These proteins are shown herein to be significantly smaller than the previously known Cry proteins in the art, and are expressed during the vegetative stage of growth of the isolated and purified bacterial cell cultures. This is unlike the expression of Cry proteins which are expressed generally in the sporulation phase of growth and which form various crystalline bodies within the forespore of the cell.

[Para 78] As will become apparent to those of skill in the art, the inventors herein disclose the isolation and purification of a nucleotide sequence, *tic*900, encoding a precursor TIC900 protein (TIC900p) that is subsequently processed to release a mature TIC900 protein (TIC900m) that exhibits lepidopteran species inhibitory biological activity. The inventors herein disclose the use of the *tic*900 sequence as a means for identifying a multitude of other homologs and related sequences, which each also encode insecticidal proteins related to TIC900.

[Para 79] Nucleotide sequences disclosed herein and encoding TIC900 and related proteins were derived from various strains of *Bacillus thuringiensis*, i.e., the strain EG5438 contained at least one gene designated herein as *tic*900. The strain EG5438 was deposited under the provisions of the Budapest Treaty with the permanent collection of the NRRL on May 3, 2002 and was provided with the NRRL accession No. NRRL B-30584. Another strain identified herein to contain a sequence encoding TIC900, a nucleotide sequence identical to the EG5438 *tic*900 allele, was *B. thuringiensis* strain EG5526.

[Para 80] Nucleotide sequences related to *tic*900, and amino acid sequences related to TIC900 (including precursor and mature species of TIC900) which are disclosed herein include but are not limited to *tic*402 and the encoded insecticidal protein TIC402 isolated from and produced at least by *B.t.* strains EG3879, *tic*403 and the encoded insecticidal protein TIC403 isolated from and produced at least by *B.t.* strain EG4332, *tic*404 and the encoded insecticidal protein TIC404 isolated from and produced at least by *B.t.* strain EG4971, *tic*434 and the encoded insecticidal protein TIC434 isolated from and produced at least by *B.t.* strain EG4611, *tic*961 and the encoded insecticidal protein TIC961 isolated from and produced at least by *B.t.* strain EG4090, *tic*962 and the encoded insecticidal protein TIC962 isolated from and produced at least by *B.t.* strain EG4293, *tic*963 and the encoded insecticidal protein TIC963 isolated from and produced at least by *B.t.* strain EG4611, *tic*965 and the encoded insecticidal protein TIC965 isolated from and produced at least by *B.t.* strain EG5023, and *tic*966 and the encoded insecticidal protein TIC966 isolated from and produced at least by *B.t.* strain EG4092.

[Para 81] It is intended that the proteins of the present invention be used for agricultural purposes, i.e., for protecting plants from insect pest infestation, and more particularly for protecting plants from lepidopteran insect pest infestation. As exemplified herein, the proteins of the present invention are useful for protecting plants at least from European corn borer (ECB) infestation, at least

from tobacco budworm (TBW) infestation and at least from diamondback moth (DBM) infestation. Plant protection can be achieved by topical application of a plant or plant parts such as by applying to the surface of the plant, i.e., the leaves, flowers, stems, stalks, and roots, a composition that contains an insecticidally effective amount of one or more of the proteins of the present invention. Alternatively, and preferably, the plant itself will be transformed to contain a nucleotide sequence modified for improved expression of the protein of the present invention *in planta* or expression of an insecticidal portion thereof.

[Para 82] The TIC900 protein is an insecticidal compound active against lepidopteran insects such as ECB, TBW and DBM. The TIC900 protein as set forth in SEQ ID NO:4 and related insecticidal proteins may be used as the active ingredient in insecticidal formulations useful for controlling lepidopteran insects. As used herein and with reference to insecticidal proteins that are related to TIC900, it is intended that related insecticidal proteins are those that are identified as homologs of TIC900 or those that are identified as being encoded by a nucleotide sequence that hybridizes under stringent conditions to all or a part of the native *Bacillus thuringiensis* sequence encoding the TIC900 protein or an insecticidal portion thereof. Of course, one skilled in the art will recognize that, due to the redundancy of the genetic code, many other sequences are capable of encoding such related proteins, and those sequences, to the extent that they function to express insecticidal proteins either in *Bacillus* strains or in plant cells, are intended to be encompassed by the present invention, recognizing of course that many such redundant coding sequences will not hybridize under stringent conditions to the native sequence encoding TIC900. Coding sequences are conceivable that function to encode all or an insecticidal portion of a TIC900 or related protein that do not hybridize under stringent conditions. However, such sequences are derived from the native nucleotide sequence on the basis that the native nucleotide sequence is capable of being modified to exhibit a non-native sequence that still encodes the same or substantially the same native amino acid sequence, or that the native amino acid sequence is capable of being used along with a codon table to back-translate, allowing the skilled artisan to arrive at a nucleotide sequence that encodes all or an insecticidal portion of a TIC900 or related protein. All of these sequences are intended to be within the scope of the present invention.

[Para 83] The *B. thuringiensis* strains containing a nucleotide sequence encoding a TIC900 or related protein and substantial equivalents thereof, can be cultured using standard known media and fermentation techniques. Upon completion of the fermentation cycle, the bacteria expressing TIC900 or a homolog thereof can be harvested by first separating the *B. thuringiensis* spores and crystals from the spent fermentation broth by means well known in the art. The recovered *B. thuringiensis* spores and crystals can be formulated into a wettable powder, a liquid concentrate, granules or other formulations by the addition of surfactants, dispersants, inert carriers and other components to facilitate handling and application for particular target pests. The formulation and application procedures are all well known in the art. The proteins in the spent fermentation broth including TIC900 or related proteins of the present invention can be concentrated and formulated into a wettable powder, a liquid concentrate, granules or other formulations by the addition of surfactants, dispersants, inert carriers and other components to facilitate handling and application for particular target pests.

[Para 84] Formulated bait granules containing an attractant and spores and crystals of the *B. thuringiensis* isolates or concentrated spent fermentation media or insecticidal proteins purified from the spores or spent fermentation media, or recombinant microbes comprising the nucleotide sequences encoding TIC900 or related insecticidal proteins obtainable from the *B. thuringiensis* isolates disclosed herein, can be applied to the environment of the pest. The bait may be applied liberally since the toxin does not affect animals or humans. Product may also be formulated as a spray or powder. Pests pick the product up on their feet or abdomen and carry it back to the nest where other pests will be exposed to the toxin. The *B. thuringiensis* isolate or recombinant host expressing a

nucleotide sequence or gene encoding a TIC900 or related protein of the present invention may also be incorporated into a bait or food source for the pest.

[Para 85] As would be appreciated by a person skilled in the art, the pesticidal concentration will vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The pesticide will be present in at least 1% by weight and may be 100% by weight. The dry formulations will have from about 1-95% by weight of the pesticide while the liquid formulations will generally be from about 1-60% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^4 cells/mg or from about 5 to about 100 parts per million of the active component insecticidal protein, i.e., the TIC900 protein, amino acid sequence variant thereof, insecticidal portion or fragment thereof, or homolog thereof. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare. The formulations can be applied to the environment of the lepidopteran pests, e.g., plants, soil, or water by spraying, dusting, sprinkling, or the like, and can also be applied to the surfaces of seeds as a seed treatment or seed coating and can be permeated into the seed coat and/or cotyledon(s).

[Para 86] One skilled in the art would know that to achieve improved expression of a Bt insecticidal protein in a plant, a nucleotide sequence encoding the Bt protein, or an active variant or fragment of the protein, would first need to be prepared. Then the nucleotide sequence encoding the protein or fragment thereof would be placed into an expression cassette that functions in plants to cause the transcription of the coding sequence into a messenger RNA that is subsequently translated in the cells of the plant such that an insecticidally effective amount of the insecticidal protein is produced within the plant tissues. One skilled in the art would also know to transform a plant cell, preferably a corn, cotton, soybean, canola, rice, wheat, oat, grass, forage plant, cruciferous plant, fruit tree, ornamental flower, tomato, potato, carrot, kale, and tobacco plant cell and the like with the nucleotide sequence embedded within the plant functional expression cassette, and to select for cells that contain the sequence and are expressing insecticidally effective amounts of the insecticidal protein, preferably a TIC900 or related protein or insecticidal fragment thereof, and to produce plants from such transformed cells. One skilled in the art would know to use electroporation, infusion, ballistic methods, or *Agrobacterium tumefaciens* mediated methods and the like for introducing the nucleotide sequences of the present invention or modifications thereof into a plant cell.

[Para 87] The term "variant or modified", with reference to nucleotide sequences, is intended to refer to nucleotide sequences which encode the same toxins or which encode equivalent toxins having similar insecticidal activity, the term "equivalent toxin" referring to a toxin exhibiting the same, essentially the same, or improved biological activity against the target pests as the claimed native or referent toxin. A variant or modified nucleotide sequence intended for use in dicot plants would encode substantially the same amino acid sequence as the native coding sequence, i.e., the coding sequence found in nature, but would comprise a total combined GC composition from about 49 to about 58 percent, and would utilize substantially the codon preference and codon usage frequency determined by compiling such preference and usage frequencies from a consortium of coding sequences derived from one or more individual dicot plant species intended to be transformed with the variant or modified nucleotide sequence. A variant or modified nucleotide sequence intended for use in a monocot plant would also encode substantially the same amino acid sequence as the native coding sequence, but would comprise a total combined GC composition from about 52 to about 59 percent, and would also utilize substantially the codon preference and codon usage frequency determined by compiling such preference and usage frequencies from a consortium of coding sequences derived form one or more individual monocot plant species intended to be transformed with the variant or modified nucleotide sequence. Codon usage frequency is intended to refer to the number of times, on average, that a particular codon is used in a coding sequence. For a particular plant species, a codon that is intended to cause the incorporation of a particular amino acid into a nascent amino acid sequence will be utilized on average with some relative fixed frequency. For

amino acids that utilize only two codons, this frequency is generally about fifty-fifty, i.e., each codon being used about half the time, unless one of the codons utilizes a substantially greater number of purines or pyrimidines that are not typically representative of the GC content of the particular plant species. For *Bacillus* species, for example, coding sequences generally are from about 60 to about 70 per cent AT. Codon usage in *Bacillus* species is biased toward the use of codons that are enriched for the presence of A or T in a particular codon. Therefore, codons that primarily utilize G or C are used in a native and/or naturally occurring *Bacillus* coding sequence with much less frequency than codons that contain A's or T's. Therefore, when producing a variant or modified nucleotide sequence intended for use in a particular plant, monocot or dicot, it is important to ensure that appropriate attention is given to the use of codons that are not particularly enriched with A's and T's where possible, and to avoid the incorporation of suspected polyadenylation sequences (see for example, US Patent No. 5,500,365).

[Para 88] As used herein, "synthetic coding sequences" or "non-naturally occurring coding sequences" encoding the *B. thuringiensis* TIC900 proteins or homologs or derivatives thereof as insecticidal toxins of the present invention are those prepared in a manner involving any sort of genetic isolation or manipulation. This includes isolation of the coding sequence from its naturally occurring state, manipulation of the coding sequence as by modification of the nucleotide coding sequence (as described herein), chemical synthesis of all or part of a coding sequence using phosphoramidite chemistry and the like, or site-specific mutagenesis (as described herein), truncation of the coding sequence or any other manipulative or isolative method so that the amino acid sequence encoded by the non-naturally occurring coding sequence encodes substantially the same insecticidal protein as the native coding sequence and furthermore exhibits substantially the same or an improved level of insecticidal bioactivity as the native insecticidal toxin protein.

[Para 89] As used herein, the phrase "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. A sequence that is identical at every position in comparison to a reference sequence is said to be identical to the reference sequence and vice-versa. A first nucleotide sequence when observed in the 5' to 3' direction is said to be a "complement" of a second or reference nucleotide sequence observed in the 3' to 5' direction if the first nucleotide sequence exhibits complete complementarity with the second or reference sequence. As used herein, nucleic acid sequence molecules are said to exhibit "complete complementarity" when every nucleotide of one of the sequences read 5' to 3' is complementary to every nucleotide of the other sequence when read 3' to 5'. A nucleotide sequence that is identical at every position when read 5' to 3' in comparison to a reference nucleotide sequence read 5' to 3' is said to be identical to the reference sequence and vice-versa. A nucleotide sequence that is complementary to a reference nucleotide sequence will exhibit a sequence identical to the reverse complement sequence of the reference nucleotide sequence. These terms and descriptions are well defined in the art and are easily understood by those of ordinary skill in the art.

[Para 90] As used herein, "substantial homology", with reference to nucleic acid sequences, refers to nucleotide sequences that hybridize under stringent conditions to the TIC900 coding sequence as set forth in SEQ ID NO:3 or complements thereof. Sequences that hybridize under stringent conditions to SEQ ID NO:3 or complements thereof, in particular from the nucleotide sequence from about nucleotide position 1 to about nucleotide position 1806, and more particularly from about nucleotide position 121 to about nucleotide position 1806, contain one or more linear

sequences that are sufficiently identical to one or more linear sequences of SEQ ID NO:3 such that an alignment is able to take place and the two sequences are then able, under stringent conditions, to form hydrogen bonds with corresponding bases on the opposite strand to form a duplex molecule that is sufficiently stable under the stringent conditions for a long enough period of time to be detectable using methods well known in the art. Such homologous sequences are from about 67% identical, to about 70% identical, to about 80% identical, to about 85% identical, to about 90% identical, to about 95% identical, to about 99% identical or greater to the referent nucleotide sequence as set forth in SEQ ID NO:3 or the complement thereof. In addition, nucleotide sequences that encode insecticidal proteins isolatable from *Bacillus thuringiensis* strains and the like, that hybridize under stringent conditions to SEQ ID NO:3 are also envisioned to exhibit substantial homology with referent nucleotide sequences that hybridize under stringent conditions to the *tic*900 coding sequence as set forth in SEQ ID NO:3 or complements thereof. Such nucleotide sequences are referred to herein as homologs of SEQ ID NO:3 and the like and comprise SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:29, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19, and related sequences and homologues thereof.

[Para 91] With reference to polypeptide sequences, the term "substantial homology" refers to polypeptides that are about 70% homologous to, about 80% homologous to, about 86% homologous to, about 90% homologous to, about 95% homologous to, about 99% homologous to, a referent polypeptide sequence. More specifically, the inventors envision substantial homologues to be about 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, and 99 percent homologous to the referent polypeptide sequence as set forth herein in SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:30, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20.

[Para 92] With reference to the proteins of the instant application, the terms "variant amino acid sequence", or "amino acid sequence variant", or "modified amino acid sequence variant" are intended to refer to amino acid sequences that are substantially equivalent to the amino acid sequences of the present invention. For example, a protein produced by the introduction of a restriction site for convenience of molecular manipulations into a coding sequence of the present invention that results in the addition or subtraction of one or more codons without otherwise (1) disrupting the native coding sequence, (2) disrupting the native open reading frame, and (3) disrupting the insecticidal biological activity of the protein, would constitute (a) a variant amino acid sequence compared to the native insecticidal toxin, (b) an amino acid sequence variant compared to the native insecticidal toxin, or (c) a modified amino acid sequence variant compared to the native insecticidal toxin. One skilled in the art would recognize that there are other types of modifications that can be made to the amino acid sequence of the present invention without disrupting the biological activity of the protein. Insertions, deletions, and substitutions are within the scope of the present disclosure to the extent that the resulting amino acid sequence variant exhibits insecticidal activity no less than that of the native insecticidal protein. Chimeras of the proteins disclosed herein, fusions of the proteins or parts of the proteins disclosed herein, and permuteins of the proteins disclosed herein are specifically contemplated.

[Para 93] The inventors contemplate that the protein compositions disclosed herein will find particular utility as insecticides for topical and/or systemic application to field crops, grasses, fruits and vegetables, and ornamental plants. In a preferred embodiment, the bioinsecticide composition comprises an oil flowable suspension of bacterial cells that expresses a novel insecticidal protein disclosed herein. Preferably the cells are *B. thuringiensis* EG5438 or SIC9002 cells, however, any such bacterial host cell expressing the novel nucleic acid segments disclosed herein and producing a crystal protein is contemplated to be useful, such as *B. megaterium*, *B. subtilis*, *E. coli*, or *Pseudomonas* spp.

[Para 94] In another embodiment, the bioinsecticide composition comprises a water dispersible granule. This granule comprises bacterial cells that express a novel insecticidal protein disclosed herein. Preferred bacterial cells are B. thuringiensis EG5438 or SIC9002 cells, however, bacteria such as *B. megaterium*, *B. subtilis*, *E. coli*, or *Pseudomonas* spp. cells transformed with a DNA segment disclosed herein and expressing the insecticidal protein are also contemplated to be useful.

[Para 95] In a third embodiment, the bioinsecticide composition comprises a wettable powder, dust, pellet, or collodial concentrate. This powder comprises bacterial cells that express a novel insecticidal protein disclosed herein. Preferred bacterial cells are B. thuringiensis EG5438 or SIC9002 cells, however, bacteria such as *B. megaterium*, *B. subtilis*, *E. coli*, or *Pseudomonas* spp. cells transformed with a DNA segment disclosed herein and expressing the insecticidal protein are also contemplated to be useful. Such dry forms of the insecticidal compositions may be formulated to dissolve immediately upon wetting, or alternatively, dissolve in a controlled-release, sustained-release, or other time-dependent manner.

[Para 96] In a fourth embodiment, the bio-insecticide composition comprises an aqueous suspension of bacterial cells such as those described above that express the insecticidal protein. Such aqueous suspensions may be provided as a concentrated stock solution which is diluted prior to application, or alternatively, as a diluted solution ready-to-apply.

[Para 97] For these methods involving application of bacterial cells, the cellular host containing the insecticidal protein gene(s) may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the *B. thuringiensis* gene. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

[Para 98] When the insecticidal compositions comprise intact *B. thuringiensis* cells expressing the protein of interest, such bacteria may be formulated in a variety of ways. They may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other pesticidal additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

[Para 99] Alternatively, the novel TIC900 or TIC900-derived or related protein or homolog thereof may be prepared by native or recombinant bacterial expression systems in vitro and isolated for subsequent field application. Such protein may be either in crude cell lysates, suspensions, colloids, etc., or alternatively may be purified, refined, buffered, and/or further processed, before formulating in an active biocidal formulation. Likewise, under certain circumstances, it may be desirable to isolate the protein in some crystalline form and/or as spores from bacterial cultures expressing the insecticidal protein and apply solutions, suspensions, or collodial preparations of such crystals and/or spores as the active bioinsecticidal composition.

[Para 100] Regardless of the method of application, the amount of the active component(s) are applied at an insecticidally-effective amount, which will vary depending on such factors as, for example, the specific lepidopteran insects to be controlled, the specific plant or crop to be treated, the environmental conditions, and the method, rate, and quantity of application of the insecticidally-active composition.

[Para 101] The insecticide compositions described may be made by formulating the bacterial cell, crystal and/or spore suspension, or isolated protein component with the desired agriculturally

acceptable carrier. The compositions may be formulated prior to administration in an appropriate means such as lyophilized, freeze-dried, desiccated, or in an aqueous carrier, medium or suitable diluent, such as saline or other buffer. The formulated compositions may be in the form of a dust or granular material, or a suspension in oil (vegetable or mineral), or water or oil/water emulsions, or as a wettable powder, or in combination with any other carrier material suitable for agricultural application. Suitable agricultural carriers can be solid or liquid and are well known in the art. The term "agriculturally-acceptable carrier" covers all adjuvants, e.g., inert components, dispersants, surfactants, tackifiers, binders, etc. that are ordinarily used in insecticide formulation technology; these are well known to those skilled in insecticide formulation. The formulations may be mixed with one or more solid or liquid adjuvants and prepared by various means, e.g., by homogeneously mixing, blending and/or grinding the insecticidal composition with suitable adjuvants using conventional formulation techniques.

[Para 102] The insecticidal compositions of this invention are applied to the environment of the target lepidopteran insect, typically onto the foliage of the plant or crop to be protected, by conventional methods, preferably by spraying. The strength and duration of insecticidal application will be set with regard to conditions specific to the particular pest(s), crop(s) to be treated and particular environmental conditions. The proportional ratio of active ingredient to carrier will naturally depend on the chemical nature, solubility, and stability of the insecticidal composition, as well as the particular formulation contemplated.

[Para 103] Other application techniques, e.g., dusting, sprinkling, soaking, soil injection, seed coating, seedling coating, spraying, aerating, misting, atomizing, and the like, are also feasible and may be required under certain circumstances such as e.g., insects that cause root or stalk infestation, or for application to delicate vegetation or ornamental plants. These application procedures are also well known to those of skill in the art.

[Para 104] The insecticidal composition of the invention may be employed in the method of the invention singly or in combination with other compounds, including and not limited to other pesticides. The method of the invention may also be used in conjunction with other treatments such as surfactants, detergents, polymers or time-release formulations. The insecticidal compositions of the present invention may be formulated for either systemic or topical use.

[Para 105] The concentration of insecticidal composition that is used for environmental, systemic, or foliar application will vary widely depending upon the nature of the particular formulation, means of application, environmental conditions, and degree of biocidal activity. Typically, the bio-insecticidal composition will be present in the applied formulation at a concentration of at least about 1% by weight and may be up to and including about 99% by weight. Dry formulations of the compositions may be from about 1% to about 99% or more by weight of the composition, while liquid formulations may generally comprise from about 1% to about 99% or more of the active ingredient by weight. Formulations that comprise intact bacterial cells will generally contain from about 10^4 to about 10^{12} cells/mg.

[Para 106] The insecticidal formulation may be administered to a particular plant or target area in one or more applications as needed, with a typical field application rate per hectare ranging on the order of from about 50 g to about 500 g of active ingredient, or of from about 500 g to about 1000 g, or of from about 1000 g to about 5000 g or more of active ingredient.

[Para 107] Modification and changes may be made in the structure of the peptides of the present invention and DNA segments which encode them and still obtain a functional molecule that encodes a

protein or peptide with desirable characteristics. In particular embodiments of the invention, amino acid sequence variants of the proteins of the present invention are contemplated to be useful for increasing the insecticidal activity of the protein, and consequently increasing the insecticidal activity and/or expression of the recombinant transgene in a plant cell. The amino acid changes may be achieved by changing the codons of the DNA sequence.

[Para 108] Proteins that are substantially equivalent to the proteins of the instant application are intended to be biologically functionally equivalent. As used herein, the phrase "biological functional equivalents", with respect to the insecticidal proteins of the present invention, are peptides, polypeptides and proteins that contain a sequence or moiety exhibiting sequence similarity to the novel peptides of the present invention, such as a TIC900 or related protein or insecticidal fragment thereof, and that exhibit the same or similar functional properties as that of the polypeptides disclosed herein, including insecticidal activity. Biological equivalents also include peptides, polypeptides and proteins that react with, *i.e.*, specifically bind to antibodies raised against epitopes present on or within TIC900 and related proteins and that exhibit the same or similar binding or reactive activity, including both monoclonal and polyclonal antibodies.

[Para 109] It is also contemplated that the proteins of the present invention could be useful for protecting dicot plants from insect infestation. Such infestations could be the result of lepidopteran, coleopteran, dipteran, or even infestation by mites, mealworms, grubs, or a wide variety of insects that injure the plant by piercing the plant tissues and extracting the nutrients intended for plant growth and development. Modifications to the primary amino acid sequence of the proteins of the present invention could result in a protein that exhibits a host range different from that of the native protein.

[Para 110] The proteins of the present invention, because of their localization into the extracellular space when expressed by *Bacillus* strains, may be useful for targeting other proteins for localization into the extracellular space. For example, the skilled artisan would know to link a first protein that is not normally secreted into the extracellular space to a second protein that is normally secreted into the extracellular space in order to achieve the localization of the first protein into the extracellular space. The proteins of the present invention could be fused by any number of means well known in the art to one or more insecticidal toxins such as crystalline delta-endotoxins to form a chimeric protein that is targeted for secretion into the extracellular space surrounding a particular host cell. It is even envisioned that the secretion event itself could lead to the separation of the two protein parts such that two separate and distinct insecticidal proteins are released into the extracellular space surrounding a particular host cell. The two proteins could either (1) both be toxic to the same insect species but effectuate their insecticidal activity using different modes of action, or (2) each be toxic to different insect species. It is conceivable that any number of insecticidal proteins could be linked end to end to the proteins of the present invention to form multimeric chimeras that are targeted to the extracellular space surrounding a particular host cell. It is preferable, in situations in which it is contemplated that other Bt insecticidal proteins are used, that the insecticidal proteins fused to the proteins of the present invention be less than full length Cry1 proteins, more preferably merely core insecticidal toxin fragments of Cry1 proteins, Cry2A proteins, Cry3 proteins, Cry9 proteins, etc. Such "other" proteins conceivably could be green fluorescent and related proteins and variants, kinases and phosphatases for modulating cell signaling processes, nucleases, lipases, herbicide tolerance proteins expressed from genes such as *gox*, various *epsps* homologues, *bar* and homologues and the like, PhnO, NptII, Aad, and the like. All of these proteins could be used as selectable markers as well, particularly when linked to a gene encoding one or more of the proteins of the present invention, to track the presence of the genes encoding one or more of the proteins of the present invention in a plant or other host cell.

[Para 111] The proteins of the present invention could be targeted for import into a subcellular organelle. For example, a first nucleotide sequence encoding a chloroplast or plastid targeting sequence could be operably linked or fused to a second nucleotide sequence encoding an insecticidal protein of the present invention to produce a chimeric precursor protein that is targeted for insertion into the chloroplast or plastid within a plant cell. Expression of such chimeric proteins would result in the import of the proteins of the present invention into the plant chloroplast or plastid, resulting in the localization of the insecticidal toxin or insecticidal fragment thereof into the chloroplast or plastid. Additionally, a nucleotide sequence encoding one or more proteins of the present invention could be localized to the chloroplast or plastid for expression. The localization of the nucleotide sequences to the plastid or chloroplast could result in the incorporation of the nucleotide sequences into the chloroplast or plastid genome, or could result in the presence of an autonomously replicating nucleic acid sequence encoding the protein of the present invention. In either sense, the proteins of the present invention would be localized to the chloroplast or plastid. As used herein therefore, the phrase "chloroplast or plastid localized" refers to a biological molecule, either polynucleotide or polypeptide, which is positioned within the chloroplast or plastid such that the molecule is isolated from the cellular cytoplasmic milieu, and functions within the chloroplast or plastid cytoplasm to provide the beneficial insecticidal effects claimed in the instant invention. Localization of a biological molecule to the chloroplast or plastid can occur, with reference to polynucleotides, by artificial mechanical means such as electroporation, mechanical microinjection, or by polynucleotide coated microprojectile bombardment, or with reference to polypeptides, by secretory or import means wherein a natural, synthetic, or heterologous plastid or chloroplast targeting peptide sequence is used which functions to target, insert, assist, or localize a linked polypeptide into a chloroplast or plastid. In any event, localization of one or more insecticidal proteins to the chloroplast or plastid necessarily implies that the resulting plant containing cells which contain plastids that contain such insecticidal protein or proteins localized within must also exhibit normal morphological characteristics. It is not known which, if any, insecticidal protein when localized to the chloroplast or plastid, will result in the achievement of a recombinant plant exhibiting normal morphological characteristics exemplified without limitation by an absence of chlorosis, an absence of stunted or stunting of the plant physiology including but not limited to thicker than average stalks, shortened stalks or internodes, inappropriate flowering, infertility, decreased yield, etc.

[Para 112] As used herein, the phrase "operatively linked" or "operably linked" refers to nucleic acid coding segments connected in frame so that the properties of one influence the expression of the other. These phrases and groups of words can also be used to refer to amino acid sequences which exhibit some function when linked to another amino acid sequence, for example, a signal peptide when linked to a protein of interest is referred to as being operably linked to the protein of interest for the purpose of targeting the protein of interest to the secretory apparatus of the host cell in which the protein is produced.

[Para 113] For the purposes of the present invention, the word "gene" refers to a nucleotide sequence that contains an open reading frame encoding a TIC900 protein, or an insecticidal fragment thereof, or an amino acid sequence variant thereof, or a related protein homolog or insecticidal fragment thereof or amino acid sequence variant thereof that is at least operably linked to a promoter sequence and a transcription termination sequence, wherein the promoter and transcription termination sequences are functional in the host cell in which the protein is produced. As used herein, "structural gene" refers to a gene that is expressed to produce a polypeptide. A structural gene of the present invention can contain, in addition to promoter and transcription termination sequences, five prime non-translated sequences, intronic sequences, and enhancer elements that function in plants in particular, and preferably those that are derived from monocotyledonous plants such as maize plants or from dicotyledonous plants such tobacco plants or cruciferous vegetable plants that, when linked together in proper sequence with one or more coding sequences of the present invention result in improved levels of expression in particular plant tissues, and preferably result in enhanced expression in leaves and stem tissues of those plants.

[Para 114] Nucleotide sequence information provided by the present invention allows for the preparation of relatively short DNA sequences, referred to herein as probes or primers, having the ability to specifically hybridize to sequences of the selected polynucleotides disclosed herein. Such nucleic acid probes of an appropriate length are prepared based on a consideration of selected polypeptide sequences encoding the insecticidal polypeptides of the present invention, *e.g.*, a sequence such as that shown in all or a probe specific part of SEQ ID NO:3, all or a probe specific part of SEQ ID NO:5, all or a probe specific part of SEQ ID NO:7, all or a probe specific part of SEQ ID NO:9, all or a probe specific part of SEQ ID NO:29, all or a probe specific part of SEQ ID NO:11, all or a probe specific part of SEQ ID NO:13, all or a probe specific part of SEQ ID NO:15, all or a probe specific part of SEQ ID NO:17, all or a probe specific part of SEQ ID NO:19, and the like. Reference to the phrase "all or a probe specific part of" is intended to refer to a nucleotide sequence probe comprising at least from about 15 to about 50, more or less, contiguous nucleotides selected from the group of nucleotides set forth in a particular referent sequence such as SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:29, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19. The ability of such nucleic acid probes to specifically hybridize to a nucleotide sequence encoding an insecticidal polypeptide sequence lends to them particular utility in a variety of embodiments. Most importantly, the probes may be used in a variety of assays for detecting the presence of complementary sequences in a given biological sample. By reference to the term "biological sample", it is intended that any sample that contains a referent nucleotide sequence that can be detected by a probe sequence as set forth herein is a sample that contains a biological molecule selected from the group consisting of contiguous nucleotide sequences set forth herein, and therefore the sample is thus referred to as a "biological sample".

[Para 115] In certain embodiments, it is advantageous to use oligonucleotide primers. The sequence of such primers is designed using a polynucleotide of the present invention for use in detecting, amplifying or modifying a defined segment of an insecticidal protein coding sequence from *B. thuringiensis* or from *Bacillus sphaericus* and the like using thermal amplification technology. Segments of nucleotide sequences related to the polynucleotides encoding the insecticidal polypeptides of the present invention may also be isolated and characterized using thermal amplification technology and such primers.

[Para 116] To provide certain of the advantages in accordance with the present invention, a preferred nucleic acid sequence employed for hybridization studies or assays or as a primer includes sequences that are complementary to at least a 14 to 30 or more contiguous stretch of nucleotides of a polynucleotide sequence encoding all or a part of an insecticidal protein of the present invention, such as that shown in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:29, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, and SEQ ID NO:22.

[Para 117] A primer or probe size of at least 14 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over segments greater than 14 bases in length are generally preferred. In order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained, one will generally prefer to design nucleic acid molecules having *tic*900-complementary sequences and the like of 14 to 20 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, or by excising selected DNA fragments from recombinant sequences localized in plasmids or other vectors containing appropriate inserts and suitable restriction sites.

[Para 118] The present invention also contemplates an expression vector comprising a polynucleotide of the present invention. Thus, in one embodiment an expression vector is an isolated and purified DNA molecule comprising a promoter operatively linked to a coding region that encodes a polypeptide of the present invention, which coding region is operatively linked to a transcription-terminating region, whereby the promoter drives the transcription of the coding region. The coding region may include a segment encoding a *B. thuringiensis* insecticidal toxin of the present invention and a segment encoding a chloroplast or plastid targeting peptide. The DNA molecule comprising the expression vector may also contain a functional intron sequence positioned either upstream of the coding sequence or even within the coding sequence, and may also contain a five prime (5') non-translated leader sequence (i.e., a UTR or 5'-UTR) positioned between the promoter and the point of translational initiation.

[Para 119] As used herein and with reference to promoter elements, the terms "operatively linked" or "operably linked" are intended to indicate that a nucleotide sequence that contains a promoter, i.e. a genetic element that functions in a particular host cell to drive the initiation of transcription, is connected to a coding region in such a way that the transcription of that coding region is controlled and substantially regulated by that promoter. Means for operatively linking a promoter to a coding region are well known in the art. Promoters that function in bacteria are well known in the art. Exemplary and preferred promoters for the *B. thuringiensis* crystal proteins include the *sig*A, *sig*E, and *sig*K gene promoters. Alternatively, native, modified, heterologous, or recombinant promoters derived from *Bacillus thuringiensis* or other *Bacillus* species can be used for achieving expression of the proteins of the present invention in a *Bacillus* species strain.

[Para 120] Where a nucleotide sequence encoding all or an insecticidal part of a protein of the present invention is to be used to transform a plant, a promoter is selected that has the ability to drive expression of the coding sequence in that particular species of a plant. Promoters that function in different plant species are also well known in the art. Promoters useful for expression of polypeptides in plants are those that are inducible, viral, synthetic, or constitutive as described in Odell *et al.* (Nature 313:810-812, 1985), and/or promoters that are temporally regulated, spatially regulated, and spatio-temporally regulated. Preferred promoters include the enhanced CaMV35S promoters, the GBOX10 promoter, the FMV35S promoter, the rice Actin promoter, and variants and chimeras thereof. For optimum control of ECB species by expression of the proteins of the present invention in plants, for example, it is preferable to achieve the highest levels of expression of these proteins within the leaves and stems of maize plants. Substantial temporal or spatial regulation refers to the expression of a gene within a plant or plant tissue from a plant operable promoter. With reference to temporal regulation, a promoter may be regulated for expression only during specific times during plant cell or tissue or even whole plant growth and development. A promoter that is actively expressing one or more genes only during seed germination would be one example of temporal regulation. Other examples could include promoters that are actively expressing one or more genes only during times when the plant, plant cell or plant tissue is exposed to certain light intensities or during total darkness. Substantial temporal regulation refers to a promoter which is actively expressed at a certain time but which may or may not be completely suppressed at other times, such that expression may still be detected by monitoring for the presence of some indicator such as an enzyme produced from a coding sequence linked to such a promoter, or as measured by the increase or decrease in some gene products such as an mRNA produced at various times throughout plant growth, differentiation, and development and/or in response to various environmental stimuli. Substantial spatial regulation refers to the expression of a gene linked to a promoter from which expression proceeds only during growth and development of certain cells or tissues within a plant. For example, a tapetal promoter is one that is substantially spatially expressed during flower growth and development. Similarly, a leaf specific or leaf enhanced promoter would only be expected to be substantially spatially expressed from within leaf cells or leaf tissues. Substantially spatially regulated also refers to the level of expression from a particular tissue specific promoter in that particular tissue and as related to levels of expression from that or a similar promoter in other tissues,

wherein expression may also be detected in tissues other than the particular tissue in which the promoter expression is preferred, but at significantly lower expression levels as measured by the production of an enzyme produced from a coding sequence linked to the promoter or by the appearance of some detectable gene product. Promoters can also be both substantially temporally and substantially spatially regulated together and simultaneously in a coordinately regulated manner. Other promoters specifically intended to be within the scope of the present invention include but are not limited to the ubiquitin promoter, the sugarcane bacilliform DNA virus promoter, the ribulose bis-phosphate carboxylase large subunit promoter, among others.

[Para 121] Preferred intron sequences for achieving optimum expression of non-naturally occurring nucleotide sequences in monocotyledonous plants may also be included in the DNA expression construct. Such an intron is typically placed near the 5'of the mRNA within or immediately downstream of an untranslated sequence. The intron could be obtained from, but not limited to, a set of introns consisting of the maize Heat Shock Protein (HSP) 70 intron (U.S. Patent 5,424,412; 1995), the rice Act1 intron (McElroy *et al.*, Plant Cell 2:163-171, 1990), the Adh intron 1 (Callis *et al.*, Genes & Develop. 1:1183-1200, 1987), or the sucrose synthase intron (Vasil *et al.*, Plant Phys. 91:1575-1579, 1989).

[Para 122] Another element that functions to regulate or to modulate gene expression is the DNA sequence between the transcription initiation site and the start of the coding sequence, termed the untranslated leader sequence (UTL). Compilations of leader sequences have been made to predict optimum or sub-optimum sequences and generate "consensus" and preferred leader sequences (Joshi, Nucl. Acids Res. 15:9627-9640, 1987). Preferred leader sequences are contemplated to include those that comprise sequences predicted to direct optimum expression of the linked structural gene, ***i.e.*** to include a preferred consensus leader sequence that increases or maintains mRNA stability and prevents inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that from genes that are highly expressed in plants, and in particular in maize will be most preferred. One particularly useful leader is the petunia HSP70 leader.

[Para 123] Transcription enhancers or duplications of enhancers could be used to increase expression. These enhancers often are found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted in the forward or reverse orientation 5' or 3' to the coding sequence. Examples of enhancers include elements from the CaMV 35S promoter, octopine synthase genes (Ellis *et al.*, EMBO Journal 6:11-16, 1987), the rice actin gene, and promoter from non-plant eukaryotes (*e.g.*, yeast; Ma *et al.*, Nature 334:631-633, 1988).

[Para 124] RNA polymerase transcribes a nuclear genome DNA coding sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of nuclear transcribed messenger RNA (mRNA). For coding sequences introduced into a chloroplast or plastid, or into a chloroplast or plastid genome, mRNA transcription termination is similar to methods well known in the bacterial gene expression art. For example, either in a polycistronic or a monocistronic sequence, transcription can be terminated by stem and loop structures or structures similar to bacterial *rho* dependent sequences.

[Para 125] Expression constructs will typically include a coding sequence exemplified in the present invention or a derivative thereof along with a 3' end DNA sequence that functions as a signal to terminate transcription and, in constructs intended for expression from the plant nuclear genome,

allow for the 3' end polyadenylation of the resultant RNA transcript. The most preferred 3' elements are contemplated to be those from the nopaline synthase gene of *A..tumefaciens* (*nos* 3' end), the terminator for the T7 transcript from the octopine synthase gene of *A. tumefaciens*, and the pea RUBISCO synthase E9 gene (E9 3') 3' non-translated transcription termination and polyadenylation sequence. These and other 3' end regulatory sequences are well known in the art.

[Para 126] Preferred plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, *e.g.*, by Herrera-Estrella (Nature 303:209-213, 1983), Bevan (Nature 304:184-187,1983), Klee (Bio/Technol. 3:637-642, 1985).

[Para 127] The present invention discloses isolated and purified nucleotide sequences encoding insecticidal proteins derived from *Bacillus* species, and particularly from *Bacillus thuringiensis* species. In particular, the *B. thuringiensis* strains EG5438, EG3879, EG4332, EG4971, EG4090, EG4293, EG4611, EG5526, EG5023 and EG4092 are each shown herein to produce one or more soluble insecticidal proteins that are localized to culture supernatants (see Table 1).

[Para 128] Table 1. TIC900 Related Proteins and Source *B. thuringiensis* Strains

Source Bt Strain	TIC900 Related Protein
EG3879, EG5526	TIC402, (TIC964)*
EG4332	TIC403
EG4971	TIC404
EG4611	TIC434
EG4090	TIC961
EG4293	TIC962
EG4611	TIC963
EG5438#	TIC900
EG5023	TIC965
EG4092	TIC966

[Para 129] * the amino acid sequence of TIC964, obtained from strain EG5526, was deduced after nucleotide sequence analysis of a gene exhibiting homology to *tic*900, and was determined to be identical to *tic*402 obtained from strain EG3879.

[Para 130] # signifies that this strain has been deposited under conditions that assure access to the culture to authorized parties during the pendency of this patent application or patents issued therefrom.

[Para 131] The *B. thuringiensis* strains and other bacterial strains described herein may be cultured using conventional growth media and standard fermentation techniques. The *B. thuringiensis* strains harboring one or more *tic*900 or related genes may be fermented as described herein until the cultured *B. thuringiensis* cells reach the stage of their growth cycle when the TIC900 and/or related proteins are produced.

[Para 132] Subject cultures have been deposited under conditions that assure that access to the culture will be available to authorized parties during the pendency of this patent application or patents issued. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

[Para 133] TIC900 and related proteins of the present invention are produced as shown herein and secreted into the growth media during the vegetative phase of growth. Fermentations using the strains of the present invention may be continued through the sporulation stage when crystal proteins, if any, are formed along with the spores. The spores and cell debris can be separated from the supernatant by centrifugation, and the spent culture medium can be used to isolate the insecticidal proteins of the present invention. The inventors herein illustrate the method of ammonium sulfate precipitation as one means for concentrating and collecting all or most of the proteins present in the spent and clarified culture medium. However, one skilled in the art will recognize that there are a number of other means available for purifying and isolating the proteins of the present invention. Gel filtration and size exclusion chromatography are two readily available means for extracting proteins directly from the spent media. Spent media can also be desalted and the filtrate used to extract protein using ion exchange columns. Also, affinity columns, containing antibodies that bind specifically to TIC900 or related proteins can be used to purify the proteins of the present invention directly from the media.

[Para 134] The amino acid sequences of the present invention have been compared to the amino acid sequences present in commercially available protein sequence databases, and no significant homologies or similarities have been identified. Based on this analysis, the TIC900 protein and related sequences appear to be unique and form the basis for the establishment of a new and separate class of *Bacillus* insecticidal proteins because the proteins of the present invention do not exhibit any relationship to other known insecticidal proteins.

[Para 135] Modification and changes may be made in the structure of the peptides of the present invention and DNA segments that encode them and still obtain a functional molecule that encodes a protein or peptide with desirable characteristics. The biologically functional equivalent peptides, polypeptides, and proteins contemplated herein should possess from about 70% or greater sequence similarity, or from about 80% or greater sequence similarity, or from about 90% or greater sequence similarity, to the sequence of, or corresponding moiety within, the fundamental TIC900 amino acid sequence as set forth in SEQ ID NO:4, or the corresponding moiety within the amino acid sequences as set forth in SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:30, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20 and related sequences.

[Para 136] According to the present invention reference to the *tic*900 gene and encoded protein toxin, includes not only the full length sequences disclosed herein but also fragments of these sequences, natural variants, mutants, and recombinant or genetically engineered derivatives of the *tic*900 gene comprising SEQ ID NO:3. Such encoded proteins should retain essentially the same as or greater characteristic insecticidal properties than those of the TIC900 protein comprising SEQ ID NO:4. The proteins useful in the present invention may also include fusion proteins that retain the characteristic insecticidal properties essentially the same as or greater than those of the TIC900 protein. In some instances, the fusion protein may contain, in addition to the characteristic insecticidal properties of the proteins specifically exemplified herein, another insecticidal activity contributed by the amino acid sequence of the fusion partner. Alternatively, crystallographic analysis of the TIC900 protein or insecticidal variants thereof may provide a means for determining whether the protein would be a candidate for the construction of a permutein that exhibits the same or preferably greater insecticidal activity than the native TIC900 or related protein, and which preferably exhibits improved characteristics related to expression in a preferred host cell such as a plant cell.

[Para 137] It should be apparent to a person skilled in the art that nucleotide sequences encoding lepidopteran inhibitory toxins can be identified and obtained through several means. The specific

sequences exemplified herein may be obtained from the isolates deposited at a culture depository as described above. These sequences, or portions or variants thereof, may also be constructed synthetically, for example, by use of a nucleotide sequence synthesizer. Variations of coding sequences may be readily constructed using standard techniques for making point mutations. Also, fragments of these sequences can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis may be used to systematically excise nucleotides from the ends of such sequences as exemplified herein or from within the protein coding sequence. Also, nucleotide sequences that encode insecticidally active protein fragments may be obtained using a variety of restriction enzymes, endonucleases, thermal amplification methods, and the like. Proteases such as proteinase K, trypsin, chymotrypsin, pepsin, and the like may be used to directly obtain active fragments of these toxins.

[Para 138] Other toxins and nucleotide sequences encoding such toxins related to the toxins and coding sequences of the present invention can be derived from DNA obtained from *B. thuringiensis*, *B. laterosporous*, *B. sphaericus*, and related *Bacillus* species isolates using the teachings provided in the art in combination with the nucleotide sequences disclosed herein. Such toxins and nucleotides sequences that are related to the toxins and coding sequences of the present invention are deemed herein to be equivalent to the toxins and nucleotide sequences of the present invention. By "equivalent" it is meant that a protein exhibits the characteristics of the TIC900 protein, including but not limited to similar insecticidal inhibitory bioactivity, host range of insecticidal bioactivity, exhibits similar antigenic epitopes that cross react with antibodies raised against TIC900 and related proteins, exhibit a similar size relative to TIC900 and related proteins, exhibit similar expression profiles and characteristics, exhibit a propensity for seclusion to the extracellular environment when expressed in *Bacillus thuringiensis* or related bacterial species, and the like. The phrase "exhibit a propensity for seclusion to the extracellular environment" is intended to include TIC900 and related proteins including but not limited to TIC402, TIC403, TIC404, TIC434, TIC961, TIC962, TIC963, TIC965 and TIC966 that are produced by the bacterium or host cell as a precursor protein that contains an amino acid sequence linked to the insecticidal protein that functions to target the insecticidal protein to a bacterial or host cell secretory apparatus and which, upon contact with the secretory apparatus, is proteolytically cleaved by a signal peptidase, releasing the mature or insecticidal protein into the extracellular environment in the case of a gram positive microbe, at least into the periplasm in the case of a gram negative microbe, and into the endoplasmic reticulum or secretory vesicle or into a subcellular organelle such as a mitochondria or chloroplast or plastic in the case of a fungal or plant or other eukaryotic host cell.

[Para 139] There are a number of methods for identifying the presence of and obtaining equivalent insecticidal toxins related to the peptides disclosed herein. For example, antibodies to the insecticidal toxins disclosed and claimed herein can be used to identify and isolate other toxins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the toxins that are most constant within the new class of proteins and most distinct from other *B. thuringiensis* toxins. These antibodies can then be used to specifically identify equivalent toxins with the characteristic activity by immuno-precipitation, enzyme linked immuno-sorbent assay (ELISA), or Western blotting. Antibodies to the toxins disclosed herein, or to equivalent toxins, or fragments of these toxins, can readily be prepared using standard procedures in the art. The nucleotide sequences that encode these toxins can then be obtained from the microorganism or other various sources.

[Para 140] Fragments and equivalents that retain the insecticidal activity of the exemplified toxins would be within the scope of the present invention. Also, because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create these alternative DNA

sequences encoding the same, or essentially the same, toxins. These variant DNA sequences are within the scope of the present invention.

[Para 141] It is well known in the art that certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the compositions disclosed herein, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity. Such substitutions are also known in the art as conservative substitutions.

[Para 142] In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

[Para 143] It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. The greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein (U. S. Patent 4,554,101).

[Para 144] As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take the various foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

[Para 145] Peptides, polypeptides, and proteins biologically functionally equivalent to TIC900, TIC402, TIC403, TIC404, TIC434, TIC961, TIC962, TIC963, TIC965 and TIC966 include amino acid sequences containing conservative amino acid changes in the fundamental sequence shown in SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:30, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20. In such amino acid sequences, one or more amino acids in the fundamental sequence is (are) substituted with another amino acid(s), the charge and polarity of which is similar to that of the native amino acid, *i.e.* a conservative amino acid substitution, resulting in a silent change.

[Para 146] Substitutes for an amino acid within the fundamental polypeptide sequence can be selected from other members of the class to which the naturally occurring amino acid belongs. Amino acids can be divided into the following four groups: (1) acidic amino acids; (2) basic amino acids; (3) neutral polar amino acids; and (4) neutral non-polar amino acids. Representative amino acids within these various groups include, but are not limited to: (1) acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; (2) basic (positively charged) amino acids such as arginine, histidine, and lysine; (3) neutral polar amino acids such as glycine, serine, threonine, cyteine, cystine,

tyrosine, asparagine, and glutamine; (4) neutral nonpolar (hydrophobic) amino acids such as alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine.

[Para 147] Conservative amino acid changes within the fundamental polypeptide sequences of the present invention can be made by substituting one amino acid within one of these groups with another amino acid within the same group. Biologically functional equivalents of TIC900 and related sequences can have 10 or fewer conservative amino acid changes, more preferably seven or fewer conservative amino acid changes, and most preferably five or fewer conservative amino acid changes. The encoding nucleotide sequence (gene, plasmid DNA, cDNA, or synthetic DNA) will thus have corresponding base substitutions, permitting it to encode biologically functional equivalent forms of TIC900.

[Para 148] Amino acid sequence variants of TIC900 and related sequences can be made by procedures well known in the art.

[Para 149] A further method for identifying the toxins and genes of the present invention is through the use of oligonucleotide probes. These probes are essentially nucleotide sequences that hybridize under stringent hybridization conditions to the TIC900 coding sequence or a sequence related to a TIC900 coding sequence. As is well known in the art, if a probe molecule and nucleic acid sequence molecule in a sample hybridize by forming a strong enough bond between the two molecules, it can be reasonably assumed that the two molecules exhibit substantial homology. Probe binding is detected using any number of means known in the art including but not limited to fluorescence, luminescence, isotopic, immunological, surface plasmon resonance spectroscopy, and the like. Such probe analysis provides a rapid method for identifying toxin-encoding genes of the present invention. The nucleotide segments that are used as probes according to the invention can be synthesized by use of DNA synthesizers using standard procedures or by other means known in the art. These nucleotide sequences can also be used as PCR primers to amplify nucleotide sequences of the present invention or portions thereof.

[Para 150] The *tic*900 and related nucleotide coding sequences as set forth herein in SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:29, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19 may be used as hybridization probes to identify and isolate natural variants of the *tic*900 and related nucleotide coding sequences from other strains of *B. thuringiensis* or from other microorganisms. The present invention encompasses nucleotide sequences from microorganisms, where the nucleotide sequences are isolatable by hybridization with all, or part, of the *Bacillus* nucleotide sequence of the invention. Proteins encoded by such nucleotide sequences can be tested for insecticidal activity. The invention also encompasses the proteins encoded by the nucleotide sequences.

[Para 151] Antibodies to TIC900 or related proteins of the present invention may be produced using standard immunological techniques for production of polyclonal antisera and, if desired, immortalizing the antibody-producing cells of the immunized host for sources of monoclonal antibody production. Techniques for producing antibodies to any substance of interest are well known, e.g., as in Harlow and Lane (1988) and as in Goding (1986). The anti-TIC900 antibodies may be used as probes to identify *B. thuringiensis* strains or other microorganisms that produce variants of TIC900 or related proteins that are encoded by variations of a *tic*900 or related gene. The present invention encompasses proteins obtained from organisms wherein the proteins obtained cross-react with antibodies raised against one or more of the proteins of the present invention.

[Para 152] The antibodies produced in the present invention are also useful in immunoassays for determining the amount or presence of a TIC900 or related protein. Such assays are also useful in quality-controlled production of compositions containing TIC900 or related proteins of the present invention. In addition, the antibodies can be used to assess the efficacy of recombinant production of a TIC900 or related protein, as well as for screening expression libraries for the presence of TIC900 or related protein coding sequences. Antibodies are useful also as affinity ligands for purifying and/or isolating TIC900 and related proteins. TIC900 and related antigenic epitopes may be obtained by over expressing full or partial lengths of a sequence encoding all or part of a TIC900 or related protein in a preferred host cell.

[Para 153] The peptides of the present invention are primarily, though not exclusively, intended for use in plants, and in certain preferred embodiments, nucleotide sequences modified for encoding the proteins of the present invention in plants are contained within one or more plasmid vectors. Such vectors may contain a variety of regulatory and other elements intended to allow for optimal expression of the proteins of the present invention in plant cells. These additional elements may include promoters, terminators, and introns as outlined above. Any vector containing the DNA construct and any regulatory or other elements may be selected from the group consisting of a yeast artificial chromosome, bacterial artificial chromosome, a plasmid, or a cosmid, and the like. Further, the expression vectors themselves may be of a variety of forms. These forms may differ for various reasons, and will likely be comprised of varying components depending upon whether they are intended to transform a monocotyledonous plant or a dicotyledonous plant.

[Para 154] Vectors further envisioned to be within the scope of the present invention include those vectors capable of containing a *tic*900 or related nucleic acid compositions disclosed above, as well as any other DNA constructs which further comprise plant-expressible coding regions for other insecticidal proteins derived from *Bacillus* species.

[Para 155] The nucleotide sequence encoding the TIC900 insecticidal protein (SEQ ID NO:4) or encoding a related polypeptide sequence such as TIC402 (SEQ ID NO:6), TIC403 (SEQ ID NO:8), TIC404 (SEQ ID NO:10), TIC434 (SEQ ID NO:30), TIC961 (SEQ ID NO:12), TIC962 (SEQ ID NO:14), TIC963 (SEQ ID NO:16), TIC965 (SEQ ID NO:18) and TIC966 (SEQ ID NO:20) may be introduced into a variety of microorganism hosts without undue experimentation, using procedures well known to those skilled in the art of transforming suitable hosts under conditions which allow for stable maintenance and expression of the cloned genes (Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual 2nd Ed., Cold Spring Harbor Press, New York). Suitable hosts that allow for expression of the TIC900 protein (SEQ ID NO:4) and related sequences include *B. thuringiensis* and other *Bacillus* species such as *Bacillus subtilis* or *Bacillus megaterium*. Genetically altered or engineered microorganisms containing the *tic*900 gene (SEQ ID NO:3) can also contain nucleotide sequences encoding other toxin proteins present in the same microorganism; these coding sequences could concurrently produce insecticidal proteins different from the TIC900 or related proteins. In particular, it would be preferable to produce two or more different insecticidal proteins in a host cell, wherein each protein is toxic to the same insect species and each protein exhibits a mode of action different from the other(s).

[Para 156] Plant-colonizing or stem-colonizing microorganisms may also be employed as host cells for the production of a TIC900 or related protein. Exemplary microorganism hosts for *B. thuringiensis* toxin genes include the plant-colonizing microbe *Clavibacter xyli* as described by Turner et al. (1993; Endophytes: an alternative genome for crop improvement; International crop science I. International Crop Science Congress, Ames, Iowa, USA, 14-22 July 1992, pp. 555-560).

[Para 157] The toxin-encoding nucleotide sequences obtainable from the isolates of the present invention can be introduced into a wide variety of microbial or plant hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable microbial hosts, e.g., *Pseudomonas,* the microbes can be applied to the situs of the pest, where they will proliferate and be ingested by the pest. The result is a control of the pest. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin and stabilize the cell. The treated cell, which retains the toxic activity, then can be applied to the environment of the target pest.

[Para 158] Where the *tic*900 toxin gene or a related nucleotide coding sequence is introduced by means of a suitable vector into a microbial host, and the host is applied to the environment in a living state, it is advantageous to use certain host microbes. For example, microorganism hosts can be selected which are known to occupy the pest's habitat. Microorganism hosts may also live symbiotically with a specific species of pest. These microorganisms are selected so as to be capable of successfully competing in the particular environment with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

[Para 159] A large number of microorganisms are known to inhabit the habitat of pests. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Bacillus, Escherichia, Pseudomonas, Erwinia, Serratia, Klebsiella, Salmonella, Pasteurella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc,* and *Alcaligenes;* fungi, e.g., genera *Metarhizium, Bavaria, Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula,* and *Aureobasidium.*

[Para 160] A wide variety of means are available for introducing a toxin gene encoding a toxin into a microorganism host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867.

[Para 161] As mentioned above, *B. thuringiensis* or recombinant cells expressing a TIC900 or related toxin can be treated to prolong the toxin activity and stabilize the cell. The pesticide microcapsule that is formed comprises one or more TIC900 or related toxins within a cellular structure that has been stabilized and will protect the toxin or toxins when the microcapsule is applied to the environment of the target pest. Suitable host cells may include either prokaryotes or eukaryotes, normally being limited to those cells that do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxic substances are unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. Of particular interest as hosts will be prokaryotes as well as lower eukaryotes such as fungi. The cells of these organisms will usually be intact and be substantially in the proliferative form when treated, rather than in a spore form, although in some instances spores may be employed. Such microcapsules can also contain one or more TIC900 or related proteins along with one or more unrelated insecticidal protein compositions including but not limited to delta endotoxins insecticidal to lepidopteran species such as Cry1, Cry2, and Cry9 proteins, as well as delta endotoxins insecticidal to coleopteran species such as Cry3, Cry22, ET70, ET80/76, ET33/34, PS149B1, ET100/101, and ET29 proteins and the like.

[Para 162] The cells generally will have enhanced structural stability that will enhance resistance to environmental conditions. Where the pesticide is in a proform or precursor form, the method of cell treatment should be selected so as not to inhibit processing of the proform to the mature form of the pesticide by the target pest pathogen. For example, formaldehyde will crosslink proteins and could inhibit processing of the proform of a polypeptide pesticide. The method of cell treatment retains at least a substantial portion of the bio-availability or bioactivity of the toxin.

[Para 163] TIC900 and related coding sequences as set forth in SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:29, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19 and the like can be used as the basis for constructing modified nucleotide sequences for incorporation into plant cells. Even more preferable is the synthesis of a non-naturally occurring nucleotide sequence that encodes a TIC900 or related insecticidal protein or its equivalent for expression in a plant cell, the synthesis of the non-naturally occurring nucleotide sequence being based on the amino acid sequence of the native protein without reference to the native nucleotide sequence from which the native amino acid sequence was deduced. Expression of such sequences in plant cells would render a plant comprised of such cells more resistant to lepidopteran species insect attack. Genetic engineering of plants with modified sequences encoding one or more TIC900 or related proteins or a related insecticidal amino acid sequence may be accomplished by introducing the desired DNA containing the coding sequence into plant tissues or cells, using DNA molecules of a variety of forms and origins that are well known to those skilled in plant genetic engineering. Method for introducing nucleotide sequences into plants, plant cells and plant tissues are well known in the art.

[Para 164] DNA containing a modified gene encoding TIC900 or a related insecticidal protein, operatively linked to a plant functional promoter, may be delivered into the plant cells or tissues directly by a number of means including but not limited to Agrobacterium mediated transformation, plant viruses, electroporation, microinjection, vacuum infiltration, liposome fusion means, and ballistic methods. The plant promoter may be a constitutive promoter; a temporally, spatially, chemically, photosynthetically, thermally, or artificially inducible promoter; a tissue-specific promoter; or a chimeric or hybrid promoter assembled from parts of other plant functional promoters. For example, the promoter may be a cauliflower mosaic virus (CaMV) 35S promoter or a plant functional derivative thereof.

[Para 165] Native bacterial genes and coding sequences are often poorly expressed in transgenic plant cells. Plant codon usage more closely resembles that of other higher organisms than unicellular organisms, such as bacteria. Several reports have disclosed methods for improving expression of recombinant genes in plants (Murray *et al.,* 1989, Nucleic Acids Research, Vol.17:477-498; Diehn *et al.,* 1998(b), Plant Physiology, 117:1433-1443; Rocher et al., 1998, Plant Phys. 117:1445-1461). These reports disclose various methods for engineering coding sequences to represent sequences which are more efficiently translated based on plant codon frequency tables, improvements in codon third base position bias, using recombinant sequences which avoid suspect polyadenylation or A/T rich domains or intron splicing consensus sequences. While these methods for synthetic gene construction are notable, synthetic genes of the present invention for expression in particular plants are prepared substantially according to the method of Brown et al. (U. S. Patent No. 5,689,052).

[Para 166] The work described herein takes advantage of methods of potentiating *in planta* expression of TIC900 and related insecticidal proteins, which confer resistance to lepidopteran insect pathogens, by incorporation or localization of coding sequences into the nuclear, plastid, or chloroplast genome of susceptible plants. U. S. Patent No. 5,500,365 and related patents describe methods for synthesizing plant genes to achieve optimum expression levels of the protein for which

the synthesized, non-naturally occurring, synthetic, or artificial gene encodes. These methods relate to the modification of native Bt structural gene sequences to produce a coding sequence that is more "plant-like" and therefore more likely to be translated and expressed by the plant, monocot or dicot. However, the method as disclosed in Brown et al. (U. S. Patent No. 5,689,052) provides for enhanced expression of transgenes, preferably in monocotyledonous plants.

[Para 167] Thus, the amount of a gene coding for a polypeptide of interest, e.g. a TIC900 or related polypeptide, can be increased in plants by transforming those plants using transformation methods mentioned above. In particular, chloroplast or plastid transformation can result in desired coding sequences being present in up to about 10,000 copies per cell in tissues containing these subcellular organelle structures (McBride et al., WO 95/24492).

[Para 168] DNA encoding TIC900 and related proteins can also be introduced into plants by utilizing a direct DNA transfer method into pollen as described (Zhou et al., 1983, Mol. Cell Biol., 10:4529-4537; Hess, 1987, Hess, Intern Rev. Cytol., 107:367.). Expression of polypeptide coding sequences, i.e., *tic*900 and the like, can be obtained by injection of the DNA into reproductive organs of a plant as described (Pena et al., 1987, Nature, 325:274). The DNA can also be injected directly into the cells of immature embryos and into rehydrated desiccated embryos as described (Neuhaus et al., 1987, Theor. Appl. Genet., 75:30).

[Para 169] After effecting delivery of exogenous nucleotide sequences encoding TIC900 or related proteins to recipient cells, the next step to obtain a transgenic plant generally concerns identifying the transformed cells for further culturing and plant regeneration, i.e., selection of the transformed cells. As mentioned herein, in order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. In this case, one would then generally assay the potentially transformed cell population by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

[Para 170] An exemplary embodiment of methods for identifying transformed cells involves exposing the transformed cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells that have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing. One example of a preferred marker gene confers resistance to the herbicide glyphosate. When this gene is used as a selectable marker, the putatively transformed cell culture is treated with glyphosate. Upon exposure to glyphosate, transgenic cells containing a recombinant GOX enzyme or a recombinant glyphosate insensitive EPSPS enzyme will be available for further culturing while sensitive, or non-transformed cells, will not. (U. S. Patent No. 5,569,834). Another example of a preferred selectable marker system is the neomycin phosphotransferase (*npt*II) resistance system by which resistance to the antibiotic kanamycin is conferred, as described in U. S. Patent No. 5,569,834. Again, after transformation with this system, transformed cells will be available for further culturing upon treatment with kanamycin, while non-transformed cells will not. Yet another preferred selectable marker system involves the use of a gene construct conferring resistance to paromomycin. Use of this type of a selectable marker system is described in U. S. Patent No. 5,424,412. Other selectable markers are well known in the art, including but not limited to antibiotic resistance markers such at *npt*II, *tet*, *aad*, and the like, *phn*O and other various acetylases (US Patent No. 6,448,476), various esterases (6,107,549), barnase (Hartley, 1988), J. Mol. Biol. 202: 913), bacterial enzymes conferring glyphosate oxidase activity upon the transformed cell (*gox*) (Barry et al., 1992, Inhibitors of amino acid biosynthesis: Strategies for imparting glyphosate tolerance to crop plants. In: Biosynthesis and Molecular Regulation of Amino Acids in Plants. pp. 139-145. Singh, Flores, and Shannon Eds., American Society of Plant Physiologists, Rockville, Md.) and the like.

[Para 171] Transplastonomic selection (selection of plastid or chloroplast transformation events) is simplified by taking advantage of the sensitivity of chloroplasts or plastids to spectinomycin, an inhibitor of plastid or chloroplast protein synthesis, but not of protein synthesis by the nuclear genome encoded cytoplasmic ribosomes. Spectinomycin prevents the accumulation of chloroplast proteins required for photosynthesis so spectinomycin resistant transformed plant cells may be distinguished on the basis of their difference in color: the resistant, transformed cells are green, whereas the sensitive cells are white, due to inhibition of plastid-protein synthesis. Transformation of chloroplasts or plastids with a suitable bacterial *aad* gene, or with a gene encoding a spectinomycin resistant plastid or chloroplast functional ribosomal RNA provides a means for selection and maintenance of transplastonomic events (Maliga, 1993, Trends in Biotechnology 11:101-106).

[Para 172] It is further contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as glyphosate or kanamycin, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and non-transformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as glyphosate or AMPA (amino-methyl phosphoric acid) at concentrations below those that cause 100% inhibition, followed by screening of growing tissue for expression of a screenable marker gene such as kanamycin would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. It is proposed that combinations of selection and screening may enable one to identify transformants in a wider variety of cell and tissue types.

[Para 173] The development or regeneration of plants from either single plant protoplasts or various explants is well known in the art. This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

[Para 174] The development or regeneration of plants containing a foreign, exogenous gene that encodes a TIC900 or related polypeptide introduced into the plant genome by *Agrobacterium* transformation of leaf explants can be achieved by methods well known in the art (Horsch *et al.*, Science 227:1229-1231; 1985). In this procedure, transformants are cultured in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant strain being transformed as described (Fraley *et al.*, PNAS, USA 80:4803; 1983). In particular, U. S. Patent No. 5,349,124 details the creation of genetically transformed lettuce cells and plants resulting therefrom which express hybrid crystal proteins conferring insecticidal activity against Lepidopteran larvae to such plants.

[Para 175] Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants, or pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important, preferably inbred lines. Conversely, pollen from plants of those important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a nucleotide sequence encoding a desired TIC900 or related polypeptide is cultivated using methods well known to one skilled in the art.

[Para 176] A transgenic plant of this invention thus has an increased amount of a coding region encoding a TIC900 or related polypeptide. A preferred transgenic plant is an independent segregant and can transmit that gene and its activity to its progeny. A more preferred transgenic plant is homozygous for that gene, and transmits that gene to all of its offspring on sexual mating. Seed from a transgenic plant may be grown in the field or greenhouse, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines that are evaluated for increased expression of the *B. thuringiensis* transgene. To identify a transgenic plant expressing high levels of a TIC900 or related protein from a preferred nucleotide sequence, it is necessary to screen the selected transgenic event, (R_0 generation) for insecticidal activity and/or expression of the gene. This can be accomplished by various methods well known to those skilled in the art, including but not limited to: 1) obtaining small tissue samples from the transgenic R_0 plant and directly assaying the tissue for activity against susceptible insects, e.g., European corn borer (ECB), tobacco budworm (TBW) and diamondback moth (DBM), in parallel with tissue derived from a non-expressing, negative control plant; 2) analysis of protein extracts by enzyme linked immunoassays (ELISA) specific for the TIC900 or related protein; or 3) reverse transcriptase thermal amplification (also known in the art as rtPCR) to identify events expressing the sequence encoding the TIC900 or related protein.

[Para 177] The following examples further illustrate the characteristics of the nucleotide sequences disclosed herein and the insecticidal activity of the proteins encoded by the disclosed nucleotide sequences. In addition, methods and procedures for practicing the invention are disclosed. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

[Para 178] Examples

[Para 179] Example 1. Preparation and bioassay of *B. thuringiensis* strain EG5438 culture supernatant

[Para 180] *B. thuringiensis* strain EG5438 was grown in 60 ml of PYG culture medium with shaking overnight at 30°C. PYG medium contained the following: 11.8 g peptone, 23.6 g yeast extract, 4 ml glycerol, 19.4 g K_2HPO_4 anhydrous, and 2.2 g KH_2PO_4 anhydrous. Deionized water was added to 1 liter, and the medium was autoclaved for 15 min. The *B. thuringiensis* culture was centrifuged at 11,000xg for 30 min and the supernatant was transferred to a clean flask. The supernatant was chilled to 4°C, and 34 grams of ammonium sulfate plus 1 ml of 1 M NaOH were slowly added to the supernatant while stirring. The mixture was centrifuged and the resulting pellet was dissolved in 2 ml of 20 mM Tris-HCl pH 7.5. The solution was transferred to dialysis tubing (6000 MWCO) and was dialyzed at 4°C against 20mM Tris-HCl pH 7.5. This is referred to as the dialyzed supernatant.

[Para 181] The dialyzed supernatant was tested for toxicity to diamondback moth (DBM) larvae as follows. Fifty µl of the dialyzed supernatant was applied topically to 2 ml of insect diet in a cup. A total of thirty-two diet cups were treated with the dialyzed supernatant. As a control sixty-four diet cups were not treated with dialyzed supernatant. One first-instar DBM larva was placed in each diet cup and insect mortality was scored after 7 days. For larvae on the untreated control diets 1 larvae out of 64 (2%) died. For larvae on the diets treated with the dialyzed supernatant 29 out of 32 (90%) died, suggesting that the dialyzed supernatant of strain EG5438 contained one or more factors toxic to DBM larvae.

[Para 182] Example 2. Fractionation of proteins in the dialyzed supernatant and bioassay of protein fractions

[Para 183] Proteins in the dialyzed supernatant were initially fractionated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Thirty µl of the dialyzed supernatant was mixed with 15 µl of protein solubilization buffer, the mixture was heated to 100°C for 5 min, and 25 µl of the mixture was applied to a polyacrylamide gel. An electric current was applied to the gel to size-separate proteins into the gel. The proteins were visualized after electrophoresis by staining with Coomassie dye. The dialyzed supernatant contained approximately twenty proteins ranging in size from approximately 20 kDa to about 100 kDa.

[Para 184] Proteins in the dialyzed supernatant were fractionated by DEAE ion-exchange chromatography. Two ml of the dialyzed supernatant was applied to a 1 ml DEAE column. The column was washed with 10 ml of 20 mM Tris-HCl, pH 7.5, followed by washing with 20 ml of a 0 to 1 M NaCl gradient in 20 mM Tris-HCl, pH 7.5. Fractions of 1 ml were collected. Each fraction was dialyzed against 20 mM Tris-HCl, pH 7.5, and individual fractions were tested for toxicity to DBM larvae as described above. Fractions with the highest toxicity were collected and combined and referred to as the DEAE pool.

[Para 185] The DEAE pool was applied to a carboxymethyl cellulose (CM) ion exchange column. The column was washed with 10 ml of 20 mM Tris-HCl, pH 7.5, followed by washing with 20 ml of a 0 to 1 M NaCl gradient in 20 mM Tris-HCl, pH 7.5. One ml fractions were collected and dialyzed against 20 mM Tris-HCl, pH 7.5. These fractions were referred to as the CM fractions. The CM fractions were tested for toxicity to DBM larvae. This analysis showed that CM fractions had the highest toxicity to DBM and contained a protein of approximately 66 kDa. The 66 kDa protein was referred to as the 5438-66 protein, also referred to as secreted TIC900, TIC900s, or mTIC900 (referring to a mature form of the protein identified in the culture supernatant that may be different from any precursor TIC900 protein (pTIC900) not yet released from the cell).

[Para 186] Example 3. Determination of the N-terminal sequence of a fragment of a TIC900 protein

[Para 187] mTIC900 protein was purified from the supernatant of strain EG5438 by DEAE and CM ion exchange chromatography. Attempts to determine the N-terminal sequence of the purified mTIC900 protein by standard methods were not successful. To overcome this difficulty, mTIC900 protein was fragmented by cyanogen bromide treatment (Cordoba et al., J. Biochem. Biophys. Methods 35: 1, 1997). The cyanogen bromide-generated TIC900 fragments were size-separated by SDS-PAGE without Coomassie staining. Separated TIC900 fragments were transferred from the SDS-PAGE to a polyvinylidene difluoride (PVDF) membrane by an electro transfer. The PVDF membrane was stained briefly with Coomassie dye and a portion of the membrane containing an approximately 14 kDa fragment of TIC900 protein was excised with a razor blade. The excised PVDF membrane containing the 14 kDa fragment was subjected to automated Edmund sequencing, revealing the amino acid sequence as shown in SEQ ID NO:1, in which the Xaa amino acid residue at position one (1) was indeterminable except that it was presumed to be either a Serine, Tyrosine, Aspartate or Histidine residue, but most likely a Tyrosine residue, Xaa amino acid residue at position 15 was indeterminable except that it was likely a Proline residue, and the Xaa residue at position 18 was also indeterminable except that it was likely an Arginine residue.

[Para 188] Example 4. Cloning tic900 gene encoding TIC900 protein

[Para 189] Based on the sequence obtained from the 14 kDa TIC900 protein fragment (SEQ ID NO:1), a gene-specific oligonucleotide was designed. Due to the degeneracy of the genetic code it is not possible to know the exact sequence of a gene based on the sequence of the protein encoded by the gene. Therefore for amino acids that can be encoded by more than a single codon, it is necessary to guess at the correct codon. The chance of guessing accurately is improved by the fact that the *B. thuringiensis* genome is approximately 68% AT (adenosine and thymidine). Therefore, for amino acids encoded by more than one codon, the codon or codons which contain A's and T's are selected, and for codons that contain substantially G/C, those codons that have a degeneracy in the third base position are selected preferentially based on whether the third base is an A or a T nucleotide. An oligonucleotide designated WD470 (SEQ ID NO:2) was designed which is one of many that could conceivably encode the amino acid set forth in SEQ ID NO:1, taking into consideration the A/T usage in *Bacillus thuringiensis* for codons encoding any given amino acid.

[Para 190] DNA was purified from *B. thuringiensis* strain EG5438 cells by standard procedures. Samples of the EG5438 DNA were subjected to either *Hind*III or *Eco*RI restriction enzyme digestion and were size-fractionated by electrophoresis through an agarose gel and subjected to Southern blot analysis using an alkaline phosphatase conjugated WD470 oligonucleotide probe. After incubation for approximately 16 hours at 40°C the blot was washed, treated with chemiluminescent buffer, and exposed to x-ray film. The WD470 probe specifically hybridized with EG5438 DNA restriction fragments that were approximately 2.5 kb (*Hind*III) and 3.0 kb (*Eco*RI) in length, respectively.

[Para 191] A library of EG5438 DNA consisting of about 3.0 kb *Eco*RI fragments was constructed in a CIP (calf intestine phosphatase) treated *Eco*RI digested pUC18 plasmid. The library was transformed by electroporation into an E. coli XL1BLUE strain and plated to LB-ampicillin. Colonies that arose were blotted to a membrane and probed with the alkaline phosphatase conjugated WD470 oligonucleotide probe. Several positive clones were selected and plasmid DNA was obtained from each. Plasmid DNA's were digested with *Eco*RI to confirm the presence of a single *Eco*RI insert consisting of about 3.0 kb. Plasmids were also subjected to hybridization to the alk-phos conjugated WD470 probe to confirm the complementarity of the probe and inserted DNA. A single clone was selected for further analysis and was designated as plasmid pEG1398. The inserted DNA in pEG1398 was subjected to sequence analysis. A sequence containing a partial open reading frame consisting of nucleotide position 1176 through 1803 as set forth in SEQ ID NO:3 was obtained, as well as an additional 24 nucleotides beyond nucleotide 1803 (data not shown) which contained a termination codon immediately after nucleotides at position 1801-1803 as set forth in SEQ ID NO:3.

[Para 192] The complete sequence of an ORF encoding the TIC900 protein was not present within the *Eco*RI fragment cloned into plasmid pEG1398. Oligonucleotide primers specific for the 5' and 3' ends of the sequence identified therein were designed to enable the synthesis of a labeled probe for use in detecting a larger cloned fragment of EG5438 DNA that likely contained the full length ORF encoding the TIC900 protein. A digoxygenin labeled DNA probe was prepared by amplification using the primers and the inserted DNA in pEG1398 as a template. The DIG-labeled DNA was used to probe a Southern blot of EG5438 DNA that had been resolved in an agarose gel after digestion with various restriction enzymes. A *Hind*III fragment about 2.5 kb in length was identified as a fragment that could contain the full length ORF encoding the TIC900 protein.

[Para 193] A EG5438 DNA fragment of about 2.5 kb was cloned using a means similar to that described above for the about 3.0 kb *Eco*RI fragment except that the *Hind*III fragment was cloned into

a pBlueScript KS plasmid and the probe used was a DIG-labeled DNA segment consisting of a part of the open reading frame identified within the 3.0 Kb *Eco*RI fragment in the plasmid pEG1398. One plasmid containing an approximately 2.5 kb *Hind*III fragment that hybridized to the DIG-labeled *Eco*RI fragment present within pEG1398 was selected for further analysis and designated as plasmid p5438-2.5-kb-H3. The recombinant *E. coli* strain harboring p5438-2.5-H3 was designated as 5438 2.5kb H3. The DNA sequence of the 2.5 kb *Hind*III insert in the plasmid p5438-2.5-kb-H3 was determined, and translation of this sequence in all six reading frames revealed an open reading frame of 1803 nucleotides, the sequence of which is set forth in SEQ ID NO:3.

[Para 194] The ORF from nucleotide position 1 through nucleotide position 1803 as set forth in SEQ ID NO:3 is predicted to encode a protein of about 68,868 Daltons, which has been designated herein as TIC900. The amino acid sequence of the predicted precursor form of a TIC900 protein (pTIC900) deduced from the open reading frame in SEQ ID NO:3 is shown as set forth in SEQ ID NO:4. Identity and similarity comparison of the amino acid sequence of the deduced TIC900 amino acid sequence (SEQ ID NO:4) with the GenBank protein database revealed that the nearest identity was to a Cry1Ca protein exhibiting about 49% identity.

[Para 195] Example 5. Expression of a cloned *tic*900 gene in recombinant *B. thuringiensis:*

[Para 196] *B. thuringiensis* insecticidal toxin genes are often poorly expressed in recombinant *E. coli* strains. *B. thuringiensis* strain EG10650 is an acrystalliferous strain that was designed for use as a recipient strain for testing whether cloned Bt genes encode insecticidal proteins. (EG10650, NRRL Accession Number NRRL B-30217, US Patent No. 6,468,52). The TIC900 coding sequence on the cloned *Hind*III fragment in plasmid p5438-2.5kb-H3 was transferred into the *Hind*III restriction site in the *B. thuringiensis- E. coli* shuttle vector pEG597 (Baum, J. A.; Coyle, D. M.; Gilbert, M. P.; Jany, C. S.; Gawron-Burke, C., 1990 Novel cloning vectors for *Bacillus thuringiensis*; Applied and Environmental Microbiology 56 (11): 3420-3428) resulting in the construction of plasmid pMON74010 which confers chloramphenicol resistance to recipient *Bacillus* cells. Plasmid pMON74010 was transformed by electroporation into the acrystalliferous *B. thuringiensis* strain EG10650 yielding strain SIC9002. Strain EG10650 was grown as a control in PYG medium as described in Example 1. The recombinant strain SIC9002 was grown in PYG medium plus 5 ug/ml chloramphenicol. Culture supernatants were prepared as described in Example 1. Proteins in the culture supernatants were resolved by standard SDS-PAGE analysis and were visualized after staining with Coomassie brilliant blue. The SDS-PAGE analysis results revealed that strains EG10650 and SIC9002 secreted similar numbers and sizes of proteins into their respective culture supernatants with the exception that the culture supernatant of strain SIC9002 contained a protein of approximately 66 kDa which did not appear to be present in the culture supernatant of strain EG10650. This result suggested that the cloned *tic*900 open reading frame in p5438-2.5kb-H3 encoded a protein that migrated with a mass of approximately 66 kDa in SDS-PAGE gels. A discrepancy in the size of the amino acid sequence deduced from the ORF as set forth in SEQ ID NO:3 (about 69 kDa) and the observed mass by migration in SDS-PAGE suggests that the secreted form of the protein may in fact be reduced in size by about 2500 to 3000 Da. This is not unexpected since most secreted proteins exhibit some proteolytic reduction in size as they are passed through any secretion machinery. However, there is no apparent type II signal peptide present as judged from an analysis of the primary amino acid sequence of the precursor TIC900 protein (pTIC900).

[Para 197] Example 6. Bioassay of TIC900 protein produced from the cloned *tic*900 coding sequence.

[Para 198] Culture supernatants of strains EG10650 and SIC9002 were applied to the surface of insect diet as described herein above. First instar European corn borer (ECB) larvae and tobacco budworm (TBW) eggs were placed on treated diet and were allowed to develop for 1 week. Insect larvae were visually evaluated. ECB larvae and TBW larvae reared on untreated diet or on diet treated with EG10650 supernatant exhibited normal growth. In contrast, ECB larvae and TBW larvae reared on diet treated with SIC9002 supernatant exhibited significant stunting. These results suggested that the protein produced from expression of the cloned *tic*900 gene inhibited growth of ECB and TBW larvae.

[Para 199] Example 7. Identification of strains containing *tic*900 homologs

[Para 200] A DIG-labeled probe encompassing the entire open reading frame of the *tic*900 coding sequence was prepared using the following thermal amplification primers:

[Para 201] 5'-gcgctagcatgaattcaaaggaacatgattatctaaaag-3', SEQ ID NO:21,

[Para 202] and

[Para 203] 5'-cgggctcgagctattcaacaggaataaattcaattttatcc-3', SEQ ID NO:22.

[Para 204] Between one and five μg genomic DNA from a collection of Bt strains was digested to completion with *Hind*III and the resulting fragments were resolved as a smear on an agarose gel. The gel was used in a Southern blot procedure in which the resolved DNA was denatured, transferred to a nylon membrane, fixed, and exposed to the DIG labeled probe described above. Hybridization was carried out in DIG Easy Hybe (Roche) at 42°C (DIG Easy Hybe at 42°C is equivalent to a stringent 42°C hybridization with a hybridization buffer system containing 50% formamide). Moderately stringent washes were performed as follows:1) one time for 5 minutes and one time for 15 minutes at 25°C in 2X SSC, 0.1%SDS; and 2) two times for 15 minutes each at 65 °C in 0.5X SSC, 0.1% SDS.

[Para 205] Thirteen strains were identified that contained from between one and three *Hind*III fragments that hybridized to the *tic*900 probe. DNA from each of these strains was used as a template for thermal amplification of *tic*900 homologs. Primers set forth as SEQ ID NO:21 and SEQ ID NO:22 were used to amplify *tic*900 homologs using the Expand High Fidelity PCR kit (Roche). Thermal amplification reaction conditions consisted of a 50 μL volume comprising 200 μM each dNTP, 300 nM each primer, 0.1-250 ng genomic DNA template, and 2.6 units enzyme mix in 1X reaction buffer (supplied by the manufacturer with the reagents in 10X concentrate).

[Para 206] Thermal amplification cycles consisted of one cycle of 2 minutes at 94°C; ten cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 2 minutes at 72°C; followed by twenty five cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 2 minutes at 72°C, increasing each of the last twenty five cycles by 5 seconds per cycle; and a terminal extension phase of 7 minutes at 72°C at the end of the last cycle.

[Para 207] DNA from nine of the thirteen strains subjected to this thermal amplification reaction produced amplification products (amplicons) that were subsequently cloned and sequenced. The 5' and 3' end sequences of the cloned thermal amplification products were fixed by the sequences of the primers and may not be representative of the sequence of the native gene throughout the sequence established by the amplification primers. Regardless, the amplicon sequences were substantially the same as the full length native sequences expressed for analysis of insecticidal activity. One skilled in the art will realize that the amplicons can be used as probes to fish out the full-length native sequences encoding insecticidal proteins related to the TIC900 protein. The proteins encoded by the open reading frame for each thermal amplification product and the strains from which each thermal amplification product were obtained are indicated in Table 1, as shown above.

[Para 208] Variant amplification primers and multiple amplification conditions were also used to identify *tic*900 homologs from the CRW-active Bt strain EG3907. The *tic*900 homolog in EG3907 was mapped by southern blot to facilitate cloning of the open reading frame encoding this protein. The *tic*900 homolog in EG3907 had a different *Hind*III restriction pattern than that of the *tic*900 gene from EG5438. The EG3907 homolog was identified on an approximately 13 kb *Bam*HI / *Bgl*II fragment. *Bam*HI / *Bgl*II -digested EG3907 DNA was ligated into *Bam*HI-digested phage lambda GEM-11 arms. Southern blots of DNA from an additional 30 Bt strains exhibiting CRW activity in the fermentation broth identified 2 strains, EG3291, EG3388, containing DNA that hybridized to a *tic*900 probe under stringent conditions. Both of these strains exhibited identical *Hind*III restriction patterns, but these were different from the restriction pattern containing the *tic*900 sequence from strain EG5438 as set forth in SEQ ID NO:3, and different from the restriction pattern containing the homolog identified as being present in strain EG3907.

[Para 209] DNA (0.5 μg) from 132 Bt strains was dot-blotted to Nytran membranes and probed with a *tic*900 DNA probe under stringent conditions. DNA from the fifteen strains exhibiting the strongest *tic*900 hybridization signals were analyzed further. DNA from each strain was digested to completion with *Hind*III and subjected to a Southern blot procedure as described above. DNA from several strains that appeared to hybridize in the dot blots did not exhibit strong hybridization signals using the Southern blot method. 14 strains containing sequences homologous to the *tic*900 gene have been analyzed using *Hind*III Southern blots. Based on the hybridization profiles that appear using *Hind*III digestion, at least 4 different *tic*900 homologs are present in these strains.

[Para 210] The following *Bacillus thuringiensis* strains exhibit *Hind*III fragments that hybridize to a *tic*900 probe under stringent or specific hybridization conditions: EG3291, EG3388, EG3879, EG3907, EG4090, EG4092, EG4293, EG4332, EG4577, EG4611, EG4963, EG4971, EG5023, EG5438, and EG5526. These strains also produce extracellular proteins that can be evaluated for insecticidal activity. Depending on the strain selected, the hybridizing *Hind*III fragments varied in size from about 0.8 kb to about 6.3 kb. The nucleotide sequence of each fragment that hybridized to the *tic*900 probe was determined, and open reading frames were deduced from these sequences, each set forth herein as SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:29, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19. The amino acid sequence of a protein comparable in size to that of TIC900 was deduced from each of these open reading frames, as set forth respectively in SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:30, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, and SEQ ID NO:20. These deduced amino acid sequences were designated respectively as TIC402, TIC403, TIC404, TIC434, TIC961, TIC962, TIC963, TIC965, and TIC966. As set forth in Table 1, these nucleotide sequences were sourced respectively from the following *B. thuringiensis* strains: EG3879 (TIC402), EG4332 (TIC403), EG4971 (TIC404), EG4090 (TIC961), EG4293 (TIC962), EG4611 (TIC963 and TIC434), EG5023 (TIC965), and EG4092 (TIC966). An additional strain was identified that exhibited a sequence that hybridized to the *tic*900 probe. Strain EG5526 contained a *Hind*III fragment that was apparently identical in size to the *Hind*III fragment identified as encoding TIC402 from strain EG3879. DNA

sequence analysis revealed that the EG5526 fragment contained an ORF (TIC964) that was identical in sequence to that of the *tic*402 ORF. Acrystalliferous strains of *B. thuringiensis* containing plasmids encoding these cloned homologs of *tic*900 were each subjected to insect bioassay and were determined to exhibit insecticidal bioactivity.

[Para 211] There is a high degree of identity between the sequences encoding the proteins of the present invention. In fact, an alignment of the open reading frames including each of the *tic* genes reveals that none of the ORF's are less than about 97% identical to each other. An alignment of the amino acid sequences encoded by each of the ORF's also indicates that there is a high degree of identity between the proteins of the present invention. TIC962 and TIC963 are the most distantly related, but still very closely related in that they exhibit greater than a 96% identity at the amino acid sequence level. Most changes to the nucleotide sequence for any given change in any ORF in relation to a consensus sequence established based on an alignment of all of the nucleotide sequences indicates that the changes are silent in that they affect only the third base in a codon and result most often in no modification of the encoded amino acid sequence.

[Para 212] Subcultures of *B. thuringiensis* strains EG5438 containing the native *tic*900 gene, and SIC9002 containing the cloned *tic*900 coding sequence were deposited in the permanent collection of the Agricultural Research Service Culture Collection, Northern Regional Research Laboratory (NRRL), U.S. Department of Agriculture (USDA), 1815 North University Street, Peoria, IL. 61604, USA. *B. thuringiensis* strain SIC9002 was deposited on April 25, 2002 and provided with the NRRL accession number NRRL B-30582. *B. thuringiensis* strain EG5438 was deposited on May 3, 2002 and was provided with the NRRL accession number NRRL B-30584.

[Para 213] Example 8. Genes Encoding Chimeric Insecticidal Proteins

[Para 214] This example illustrates that the TIC900 class of proteins exhibit similarities with the Cry1 class of Bt insecticidal proteins and that a chimeric protein can be constructed from all or a part of a TIC900 class protein linked in frame with all or a part of a Cry1 protein and tested for insecticidal activity.

[Para 215] Comparison of any of the TIC900 class of proteins disclosed herein with other Bt insecticidal proteins suggests that these proteins are most closely related to the Cry1 classes of proteins, and in particular to the insecticidal portion of the Cry1 proteins. The TIC900 class of proteins exhibit structural similarities to the Cry1 protein toxin portions in that the Cry1 proteins exhibit a domain structure consisting of a first domain consisting of about the first 200 to about the first 240 amino terminal amino acids which is referred to as domain I, a second domain that consists of about amino acids 240 through about amino acid 400 or so which is referred to as domain II, and a carboxy-terminal domain referred to as domain III consisting of amino acids from about residue 400 or so through the end of the toxin domain. The TIC900 class of proteins appear to exhibit this type of domain structure even though the TIC900 class of proteins generally are not as long as most Cry1 toxin domains. It has previously been shown that Cry1 toxin domains can be fused to heterologous protoxin peptide structures, and that the fusions result in crystal formation, and often also retain insecticidal bioactivity when the resulting crystals are tested in bioassay. A fusion protein (SEQ ID NO:24, TIC109) was constructed in which TIC900 was fused to the Cry1Ac protoxin peptide structure. The fusion protein was expressed from the nucleotide sequence as set forth in SEQ ID NO:23 in pMON74119 in *B. thuringiensis* strain EG10650 (recombinant strain designated as SIC1047). SEQ ID NO:23 corresponds to a TIC900 coding sequence from nucleotide position 1-1809, and a Cry1Ac protoxin domain coding sequence from nucleotide position 1816 –3504. The chimeric protein TIC109 formed in SIC1047 fermentations produced crystalline inclusions, which were tested in bioassay against Tobacco Budworm, Corn Earworm, and Fall Armyworm. The

chimeric protein exhibited bioactivity similar to that exhibited by TIC900, but was not biologically active against Fall Armyworm.

[Para 216] TIC110 (SEQ ID NO:26) encoded by the nucleotide sequence as set forth in SEQ ID NO:25 is a Cry1F/TIC900 chimeric insecticidal protein linked to a Cry1Ac protoxin peptide sequence. SEQ ID NO:25 corresponds to a sequence encoding Cry1F domain I from about nucleotide position 1-723, a sequence encoding TIC900 domains II and III from about nucleotide position 724-1809, and a Cry1Ac coding sequence from about nucleotide position 1810-3510. This protein can be expressed in an acrystalliferous strain of Bt and the crystalline protein inclusions tested in bioassay to determine the biological activity against various lepidopteran pest species.

[Para 217] TIC111 (SEQ ID NO:28) is encoded by the nucleotide sequence as set forth in SEQ ID NO:27. TIC111 corresponds to an insecticidal chimeric protein consisting of a Cry1Ac domain I linked to TIC900 domains II and III, which is linked to a Cry1Ac protoxin domain. TIC111 can be expressed from pMON74122 and the crystalline protein inclusions tested in bioassay to for bioactivity against various lepidopteran pest species.

[Para 218] pMON74122 was transformed into the acrystalliferous Bt strain EG10650 resulting in the transformed host cell SIC1049 expressing the TIC111 protein. TIC111 crystals were collected and tested in bioassay against black cutworm (BCW), Diamondback Moth (DBM), Tobacco Budworm (TBW), Corn Earworm (CEW), and Fall Armyworm (FAW). Insecticidal bioactivity was observed for BCW, DBM and TBW, consistent with the insecticidal bioactivity for TIC900.

[Para 219] In summary, the above detailed description describes the present invention. It will be understood by those skilled in the art that, without departing from the scope and spirit of the present invention and without undue experimentation, the present invention can be performed within a wide range of equivalent parameters. While the present invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. The present invention is intended to include any uses, variations, or adaptations of the invention following the principles of the invention in general. Various permutations and combination of the elements provided in all the claims that follow are possible and fall within the scope of this invention.

[Para 220] Reference to the word 'comprising' or 'comprise' or 'comprises' whether in the claim language or in the specification is intended to be defined as a term or terms meaning "includes at least".

[Para 221] All publications and patents mentioned in this specification are herein incorporated by reference as if each individual publication or patent was specially and individually stated to be incorporated by reference.

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